ENDOCRINE CONTROL OF OVULATION RATE IN THE COW

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ABSTRACT

Techniques to increase prolificacy in cattle have met with limited success; the aim of these studies was to investigate the potential of actively immunising cattle against certain gonadal hormones, and to examine the physiological basis of these treatments. Eight heifers were immunised against 8mg of a testosterone conjugate in Freund's Incomplete Adjuvant, & nine animals served as controls. These heifers were given one priming and two booster injections at four-month intervals. After the last booster injection, 7/8 animals had become anoestrous, and displayed significantly raised blood progesterone & mean LH concentrations, increased LH pulse frequency, & decreased mean FSH concentrations. Seven months after this treatment, 3/7 anoestrous heifers resumed ovarian cyclicity, with a mean ovulation rate of 2.7±0.7.

To determine if the different ovarian responses observed above could be obtained by changes of ovarian steroid feedback seen during the oestrous cycle, groups of 6 heifers were implanted with large, medium or small sized oestradiol capsules during the luteal phase of the cycle. Five control heifers received empty implants. During the luteal phase of the cycle following implantation, all heifers were ovariectomised. The effect of the treatment on ovarian function and gonadotrophin secretion in the presence or absence of progesterone (PRID) was then determined. Increasing physiological concentrations of oestradiol reduced the number of large antral follicles and corpora lutea, but not the total number of antral follicles >1mm diameter. A combination of progesterone and oestradiol were fully effective in maintaining luteal-phase concentrations of LH and FSH, and follicular-phase concentrations of oestradiol alone were able to maintain LH and FSH concentrations

within the physiological range. Thus changes of blood steroid levels similar to those seen during the oestrous cycle may interrupt ovarian function.

Cattle were therefore immunised against a non-steroidal, partly purified fraction of ovine follicular fluid (PPFF) enriched in inhibin-like activity as measured *in vivo* and *in vitro*. Active immunisation against 0.4mg and 4mg ovine PPFF produced 1/5 & 3/5 heifers with multiple ovulations, respectively; this was not associated with changes of FSH secretion. To examine in more detail the endocrine responses to this treatment, and to investigate possible comparative aspects, cows were immunised against 4mg ovine, porcine or equine PPFF. No treatment increased ovulation rate, but the porcine-PPFF immunised heifers showed a 7-fold increase in mean LH secretion that could not be explained by alterations in pulsatile secretion or in steroid feedback.

Collectively, these results suggest that the cow does not respond consistently to treatments so far designed to alter gonadotrophin secretion, that inhibin is not a major feedback hormone in this species, and that the heifer may possess an influential intra-ovarian control mechanism which ultimately determines ovulation rate.

DECLARATION

I hereby declare that this thesis has been composed by myself, and has not been submitted for any other degree, in Edinburgh or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the preparation of this thesis.

Christopher A. Price

PUBLICATIONS ARISING FROM THE THESIS

1. Abstracts of spoken papers

C.A. PRICE, R.B. LAND, B.A. MORRIS, T. O'SHEA & R. WEBB. Active immunisation against 'inhibin' and testosterone in the heifer. Annual Conference of the Society for the Study of Fertility, Aberdeen, 1985.

C.A.PRICE, R.WEBB, R.B.LAND, B.A.MORRIS & T.O'SHEA. Gonadotrophin & progesterone secretion after active immunisation of the heifer against testosterone and 'inhibin'. Annual Conference of the Society for the Study of Fertility, Bristol, 1986.

C.A. PRICE, B.A. MORRIS & R. WEBB. Active immunisation of the cow against "inhibin" preparations from various species. Joint Winter Meeting of the Society for the Study of Fertility & the British Neuroendocrine Group, Nottingham, 1986.

C.A. PRICE, D. McGAVIN & R. WEBB. Steroid control of LH, FSH and ovulation rate in the cow. Submitted for the Annual Conference of the Society for the Study of Fertility, York, 1987.

2. Refereed papers

C.A.PRICE, B.A.MORRIS & R.WEBB. Reproductive and endocrine effects of active immunization against a testosterone conjugate in the heifer. J. Reprod. Fert., in press.

C.A.PRICE, B.A.MORRIS, T.O'SHEA & R.WEBB. Active immunization of cattle against partly purified follicular fluid from sheep. J. Reprod. Fert., in press.

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INTRODUCTION

Various reproductive technologies are now available to synchronise oestrus and increase conception rates, thereby improving fertility, in a range of domestic species throughout the developed world.

However, progress to improve prolificacy, the number of viable young born per gestation, has been made in only a few species. Genetic selection and immunoneutralisation of gonadal steroids have been successful in sheep but not in cattle; indeed, triplet births in the cow appear worthy of reporting in the national press (The Scotsman, November 23rd, 1985).

It is not only desirable to increase prolificacy in cattle, but also to control it; thus genetic selection techniques are no longer favoured. Instead, it is necessary to develop a treatment that can reliably induce twin births in selected cattle at the required time. The failure of current techniques to achieve this has implied that there is some fundamental difference in the reproductive physiology of the sheep and the cow. This difference may be extended to the endangered species, particularly those which are difficult to breed in captivity.

The aim of this thesis was to examine the effects of several approaches to the immunological manipulation of ovarian function in the cow, to clarify the physiological basis of these techniques, and to further our understanding of the factors which limit prolificacy in this species.

CHAPTER ONE

LITERATURE REVIEW

1.1 ENDOCRINE CONTROL OF THE BOVINE OESTROUS CYCLE

Female cattle attain sexual maturity between the ages of 5 and 15 months, depending on bodyweight (Hammond, 1927), and then undergo regular oestrous cycles until culling. Since domestic cattle are rarely, if ever, allowed to reach the end of their natural lifespan, the effects of great age on reproductive function in this species are largely unknown. Erickson (1966) has reported a decrease in the number of antral follicles in the ovaries of cows over the age of 15 years, although Krohn (1964) has reported that cattle are no less fertile at eight years than before.

The cow does not exhibit seasonal anoestrus as seen in the sheep (Goodman, Legan, Ryan, Foster & Karsch, 1981a), although seasonal variations in behavioural oestrus, and some follicular and endocrine patterns have been described (Tucker, 1982; McNatty, Hudson, Gibb, Henderson, Lun, Heath & Montgomery, 1984a; Fulkerson & Dickens, 1985; Pennington, Albright, Diekman & Callahan, 1985; Day, Imakawa, Pennel, Zalesky, Clutter, Kittok & Kinder, 1986). The major anoestrous period in the cow is pregnancy and the involutionary period post partum. The duration of pregnancy is approximately 285 days (Hammond, 1927) and the ensuing anoestrus is reported to last from 30 to 80 days or more (Morrow, Roberts & McEntee, 1969). The physiology of anoestrus post partum has received much attention (see Hanzen, 1986; Peters & Lamming, 1986), and will not be considered further in this review.

The mean length of the oestrous cycle in the cow is 21 days (Hammond, 1927; Olds & Seath, 1951; Hafez & Sugie, 1963), with 79% of all cycles falling within the range 17 to 23 days (Lasley & Bogart, 1943). The oestrous cycle can be divided into the oestrous,

metoestrous, dioestrous and pro-oestrous phases, but these tend to be indistinct in the large domestic species. It is thus more convenient to consider the cycle in two parts, namely the luteal and follicular phases. The follicular phase culminates in the ovulation of normally only one follicle, which is more often on the cows' right ovary (Reece & Turner, 1938; Rajakoski, 1960).

1.1.1 TEMPORAL CHANGES

1.1.1.1 Luteal Phase

The luteal phase is characteristically dominated by the corpus luteum, although for approximately four days after ovulation the corpus luteum is reported to be loosely organised, often filled with blood, and of immature dimensions (0.5 - 1.6cm in diameter and 0.8 - 1.0g in wet weight; Ireland, Murphree & Coulson, 1980; Milvae & Hansel, 1983). This constitutes the early luteal phase, and peripheral progesterone concentrations remain low, rarely rising above 2ng/ml before Day 5 (Wettemann, Hafs, Edgerton & Swanson, 1972; Glencross, Munro, Senior & Pope, 1973; Schams, Schallenberger, Hoffmann & Karg, 1977; Milvae & Hansel, 1983).

As well as the immature corpus luteum, many authors have identified one large (6 - 10mm diameter) follicle on the ovaries during the early luteal phase, which has the ability to secrete a large quantity of oestradiol (Ireland & Roche, 1983a; Kruip & Dieleman, 1985), an indicator of follicular health (see McNatty,

Heath, Henderson, Lun, Hurst, Ellis, Montgomery, Morrison & Thurley, 1984b). Oestradiol has been shown to be secreted into the ovarian vein in a pulsatile nature with a frequency of approximately one pulse every 2 hours, giving a mean vena cava concentration of 23pg/ml (Walters, Schams & Schallenberger, 1984). Oestradiol has been detected in the peripheral circulation (mean of approximately 5pg/ml), but pulses are not evident (Walters et al., 1984).

Pituitary luteinising hormone (LH) is also secreted in a pulsatile manner, with one pulse of mean amplitude 0.6ng/ml occurring every 1.5 - 2 hours (Rahe, Owens, Fleeger, Newton & Harms, 1980; Walters et al., 1984; Peters, 1985), giving mean concentrations ranging between 0.8 - 1.8ng/ml during the early luteal phase, depending upon the assay in use (Christensen, Hopwood & Wiltbank, 1974; Schams et al., 1977; Walters et al., 1984; Peters, 1985). These LH pulses were found to be secreted concomitantly with pituitary follicle stimulating hormone (FSH) pulses, and followed immediately by pulses of ovarian progesterone. Approximately 90% of LH pulses were followed within 60 minutes by a pulse of ovarian oestradiol secretion (Walters & Schallenberger, 1983; Walters et al., 1984). Further information on FSH pulse secretion is not available in the literature because of the lack of consistent and sensitive assays for bovine FSH and infrequent sampling regimes; mean luteal phase concentrations range from 20ng/ml to 400ng/ml depending upon the assay in use (Akbar, Reichert, Dunn, Kaltenbach & Niswender, 1974; Derivaux, Ectors, Hendrick & Franchimont, 1974; Schams & Schallenberger, 1976; Schams et al., 1977; Peters, 1985). FSH has been reported to rise above mean concentrations in synchrony with waves of follicular growth, and two such increases occur on Days 4 and 8 of the cycle

(Derivaux et al., 1974; Schams & Schallenberger, 1976; Schams et al., 1977).

The period from Day 6 - 7 to Day 10 - 12 of the cycle is the mid-luteal phase, and progesterone concentrations in the peripheral circulation increase to 5 - 6ng/ml (Wettemann et al., 1972; Glencross et al., 1973; Schams et al., 1977; Wise, Caton, Thatcher, Barron & Fields, 1982a; Milvae & Hansel, 1983), although mean concentrations as high as 12ng/ml have been reported (Peters, 1985). The corpus luteum reaches its mature size of 1.6 - 2.5cm in diameter and 3.0 - 5.5g wet weight by Day 10 - 12, becomes well vascularised on the external surface and fades slightly in colour from a bright red to orange (Hammond, 1927; Foley, Black, Black, Damon & Howe, 1964; Priedkalns, Weber & Zemjanis, 1968; Ireland et al., 1980; Milvae & Hansel, 1983; Pierson & Ginther, 1984). Mean ovarian blood flow has been shown to reach a peak between Days 10 & 12 (Wise et al., 1982a).

By Day 7 of the cycle, the large follicle has regressed and been replaced with another large 'dominant' follicle, 1 - 1.6cm in diameter (Hammond 1927), which is generally considered to be atretic (Bane & Rajakoski, 1961; Lunaas, 1964; England, Karavolas, Hauser & Casida, 1973; Ireland & Roche, 1982a, 1983a). The numbers and sizes of small follicles (less than 5mm in diameter) tend to decrease towards mid-cycle (Matton, Adelakoun, Couture & Dufour, 1981; Ireland, Coulson & Murphree, 1979), and follicles within the 5 - 8mm diameter range increase in number to reach a maximum at Day 12 -13 (Marion, Gier & Choudary, 1968; Schams et al., 1977; Matton et al., 1981). These findings are compatible with the rapid turnover of medium sized follicles at this time (Rajakoski, 1960; Choudary, Gier & Marion, 1968; Marion et al., 1968; Maurasse,

Matton & Dufour, 1985; Spicer & Echternkamp, 1986). Whilst peripheral oestradiol concentrations remain low during most of the luteal phase (2 - 5pg/ml; Wettemann et al., 1972; Glencross et al., 1973; Dobson & Dean, 1974; Glencross & Pope, 1981; Peters, 1985), an elevation above the baseline has been observed around days 6 to 9 (Glencross et al., 1973; Dobson, Cooper & Furr, 1975; Glencross & Pope, 1981), coinciding with the increase in follicular growth.

Intensive sampling of jugular blood has revealed that LH pulse frequency appears to be lower than that of the early luteal phase, at approximately one pulse every 4 - 6 hours, and with a pulse amplitude of up to 2ng/ml; mean concentrations are no different between early and mid-luteal phases (Rahe et al., 1980; Walters et al., 1984; Peters, 1985). These LH pulses occur concomitantly with FSH pulses, but separate FSH pulses have also been reported, to give a mean FSH pulse frequency of one pulse every 2 - 3 hours (Walters & Schallenberger, 1983; Walters et al., 1984; Schallenberger, Schondorfer & Walters, 1985). In several studies, gonadotrophin pulses were followed by pulses of ovarian progesterone secretion, pulsatile increases in ovarian blood flow, and, after a time lag of approximately 60 minutes, pulses of ovarian oestradiol secretion (Hixon, Pijanowski, Weston, Shanks & Wagner, 1983; Walters et al., 1984; Procknor, Dachir, Owens, Little & Harms, 1986). The separate FSH pulses have been associated with pulses of progesterone secretion in vena cava (Walters et al., 1984), but not jugular blood (Procknor et al., 1986).

Oxytocin is also secreted from the ovary and concentrations in the peripheral circulation rise to 2.5pg/ml in the mid-luteal phase (Schams, 1983). Like oxytocin, neurophysin I and vasopressin have

been found in the corpus luteum, and are present in the highest concentrations during the mid-luteal phase (Wathes, Swann & Pickering, 1984). Intensive sampling of ovarian venous blood has indicated that oxytocin and neurophysin I are secreted simultaneously in pulses every 4 - 6 hours in the early luteal phase and every 2 - 3 hours in the mid-luteal phase (Walters et al., 1984; Schams, Schallenberger & Legros, 1985). Over 97% of these pulses were associated with a pulse of progesterone (and also FSH), but only 30% of early luteal and 86% of mid-luteal progesterone pulses were associated with pulses of oxytocin (Walters et al., 1984).

In contrast to oestrogen and progesterone, the androgens do not appear to be secreted in any consistant pattern; Peterson, Fairclough & Smith (1978) reported that mean peripheral serum concentrations of androstenedione and testosterone vary between 5pg/ml & 80pg/ml at all stages of the cycle, with an elevation during the follicular phase (Shemesh & Hansel, 1974). However, Wise, Caton, Thatcher, Lehrer & Fields (1982b) demonstrated a trend for ovarian venous androstenedione concentrations to increase steadily throughout the luteal phase, and for 'spikes' of testosterone secretion from the ovary to be more frequent at luteolysis. Serum prolactin concentrations have been reported to vary between cows and to show infrequent and irregular pulses above a mean ranging from 10 - 50ng/ml. The variation between phases is reported to be less than the variation between seasons (Schallenberger, Schams, Bullerman & Walters, 1984; Walters & Schallenberger 1984; Walters et al., 1984).

After approximately Day 14 - 18, the corpus luteum starts to decrease in size (Hafez & Sugie, 1963), although progesterone concentrations remain high until Day 16 - 18 (Wettemann et al.,

1972; Glencross et al., 1973; Kindahl, Edqvist, Bane & Granstrom, 1976; Schams et al., 1977; Peters, 1985). After about Day 15 to 17, peripheral oestradiol concentrations begin to rise above baseline (Glencross et al., 1973; Peterson, Fairclough, Payne & Smith; 1975; Fogwell, Cowley, Wortman, Ames & Ireland, 1985) as the turnover of follicles greater than 5mm in diameter increases and the non-ovulatory 'dominant' follicle begins to regress (Ireland, Coulson & Murphree, 1979; Matton et al., 1981; Ireland & Roche, 1983a; Quirk, Hickey & Fortune, 1986).

The increase in serum oestradiol at the end of the luteal phase causes an increase in uterine blood flow, and is postulated to cause the observed increase in uterine secretion of the F series of prostaglandins (PGF), and a major metabolite, 13,14,dihydro-15-keto-PGF2α (PGFM; Peterson et al., 1975; Hansel, Hixon, Shemesh & Tobey, 1976; Kindahl et al., 1976; Thatcher, Wolfenson, Curl, Rico, Knickerbocker, Bazer & Drost, 1984). A proportion of the prostaglandin outflow passes into the ovarian artery from the utero-ovarian vein by way of the counter-current model of McCracken, Baird & Goding (1971; Hixon & Hansel, 1974; Krzymowski, Stefanczyk, Kotwica, Czarnocki, Glazer, Janowski & Chmiel, 1982; Wolfenson, Thatcher, Drost, Caton, Foster & LeBlanc, 1985) and thus acts directly on the corpus luteum. Evidence is available for the luteolytic effects of oestradiol (Brunner, Donaldson & Hansel, 1969; Lemon, 1975; Ely, Thatcher & Bazer, 1979; Munro & Moore, 1985; Villa-Godoy, Ireland, Wortman, Ames, Hughes & Fogwell, 1985), PGF (Lamond, Tomlinson, Drost, Henricks & Jochle, 1973; Louis, Hafs & Seguin, 1973; Stellflug, Louis, Gorewit, Oxender & Hafs, 1977; Schams & Karg, 1982) and oxytocin (Armstrong & Hansel, 1959; Milvae & Hansel, 1980). The role of

oxytocin in luteolysis has been reviewed by Wathes (1984). The luteolytic influence of the uterus on the corpus luteum results in a sharp fall in peripheral progesterone concentrations at around Day 18 (Hansel & Snook, 1970; Ireland et al., 1980; Glencross & Pope, 1981; Wise et al., 1982a; Schallenberger et al., 1984), and signals the end of the luteal phase of the oestrous cycle.

1.1.1.2 Follicular Phase

After the decline in progesterone concentrations, the corpus luteum regresses to less than 1cm in diameter and about 1g wet weight, loses its external vasculature and becomes predominantly connective tissue as it fades in colour from orange to yellow between Days 17 & 20 (Priedkalns et al., 1968; Ireland et al., 1980). The mean numbers, volumes and diameters of healthy follicles greater than 6mm in diameter increase from Day 18 until ovulation (Ireland & Roche, 1982a, 1983b; Pierson & Ginther, 1984) and the mean peripheral plasma oestradiol concentration continues to rise to 8 to 10pg/ml (Wettemann et al., 1972; Glencross et al., 1973; Peterson et al., 1975; Schams et al., 1977; Glencross & Pope, 1981; Peters, 1985), although concentrations as high as 20 -25pg/ml have been reported (Lemon, Pelletier, Saumande & Signoret, 1975; Dobson, 1978).

Mean LH concentrations rise from 1.5ng/ml after luteolysis, to 2.5ng/ml on Day 20 (Peters, 1985), and intensive blood sampling between Days 18 & 20 has shown that LH pulse frequency has risen to one pulse per hour, with a pulse amplitude of 1.7 - 2.0ng/ml (Rahe et al., 1980; Peters, 1985); values for LH pulse amplitude and

mean concentrations have been reported to be higher after PGF-induced luteolysis than those seen after spontaneous luteolysis (Imakawa, Day, Zalesky, Garcia-Winder, Kittok & Kinder, 1986). The mean circulating concentrations of FSH do not change significantly (Peters, 1985), but the pulse frequency has risen to that of LH and without any separate FSH pulses (Schallenberger et al., 1984). Elevations above the mean have been reported to occur on Days 18 and 20 (Schams et al., 1977; Walters & Schallenberger, 1983). Ovarian oestradiol pulse frequency has also risen to that of LH, but with a time lag of approximately 20 minutes (Walters & Schallenberger, 1983, 1984; Walters et al., 1984).

At around Day 18, and not before, one preovulatory follicle arises from the pool of healthy follicles greater than 6mm in diameter (Dufour, Whitmore, Ginther & Casida, 1972; Matton et al., 1981; Staigmiller & England, 1982; Quirk et al., 1986) and grows rapidly in size to approximately 16mm diameter by the onset of behavioural oestrus (England et al., 1973; Dieleman & Blankenstein, 1984, 1985). This follicle secretes large amounts of oestradiol (Ireland & Roche, 1982a; Staigmiller, England, Webb, Short & Bellows, 1982; Dieleman, Kruip, Fontijne, deJong & van der Weyden, 1983; Braden, Manns, Cermak, Nett & Niswender, 1986) and is responsible for most of the circulating oestradiol seen at this time (Ireland, Fogwell, Oxender, Ames & Cowley, 1984).

During the late follicular phase, the vaginal and cervical epithelia differentiate and comprise a large number of mucus-secreting cells (Cole, 1930). At the onset of oestrus (designated Day 0 of the cycle), a clear and fluid mucus flows from the vagina, and becomes thicker and steadily more opaque as oestrus proceeds (Hammond, 1927; Cole, 1930; Bane & Rajakoski, 1961);

behavioural oestrus lasts for 12 - 24 hours (Hafez & Sugie, 1963; Schams et al., 1977). Towards the end of oestrus, LH and FSH pulse frequencies and amplitudes rise sharply as the release of LH surges to a peak of 7 - 80ng/ml over a 4 - 5 hour period, and then declines as quickly as it rose (Christensen et al., 1974; Schams et al., 1977; Rahe et al., 1980; Walters & Schallenberger, 1984). The secretion of FSH also surges, but not as markedly as the LH surge (Akbar et al., 1974; Schams & Schallenberger, 1976; Schams et al., 1977; Dobson, 1978). A peak of oestradiol has been reported to occur at the same time as the gonadotrophin surge in both vena cava and jugular blood, and a smaller rise in progesterone has been shown in vena cava blood (Chenault, Thatcher, Kalra, Abrams & Wilcox, 1975; Walters & Schallenberger, 1984). Lemon et al. (1975) have indicated that cattle will stand to be mounted only during the period when serum LH concentrations were above baseline.

After the gonadotrophin surge, ovarian oestradiol secretion falls rapidly (Ireland et al., 1984; Fogwell et al., 1985) as the oestradiol content of the preovulatory follicle declines (Staigmiller et al., 1982; Dieleman et al., 1983). There is an absence of ovarian oestradiol and pituitary LH pulsatile release for approximately 12 hours after the gonadotrophin surge (Walters & Schallenberger, 1984). Dieleman and Blankenstein (1984, 1985) have reported that there is a marked switch in follicular steroidogenesis, from oestradiol to progesterone, at around 14 - 20 hours after the gonadotrophin surge; this corresponds to a proliferation of granulosa cells within the 18mm diameter follicle (Dieleman et al., 1983), and the resumption of LH pulse release at a frequency of one pulse every hour (Walters & Schallenberger, 1984).

Ovulation occurs between 14 - 29 hours after the gonadotrophin

surge (Hammond, 1927; Nalbandov & Casida, 1942; Schams et al., 1977; Bernard, Lambert, Beland & Belanger, 1984), or 28-31 hours after the onset of oestrus (as observed by Hunter & Wilmut, 1984), and coincides with the beginning of a secondary rise in serum FSH concentrations (Dobson, 1978; Walters & Schallenberger, 1984). The cavity left by the burst follicle soon fills with blood and becomes the 'early' corpus luteum of the next cycle (Milvae & Hansel, 1983). A diagrammatic representation of the endocrine patterns occurring in the bovine oestrous cycle is given in Figure 1.1.

1.1.2 GONADOTROPHIN SECRETION

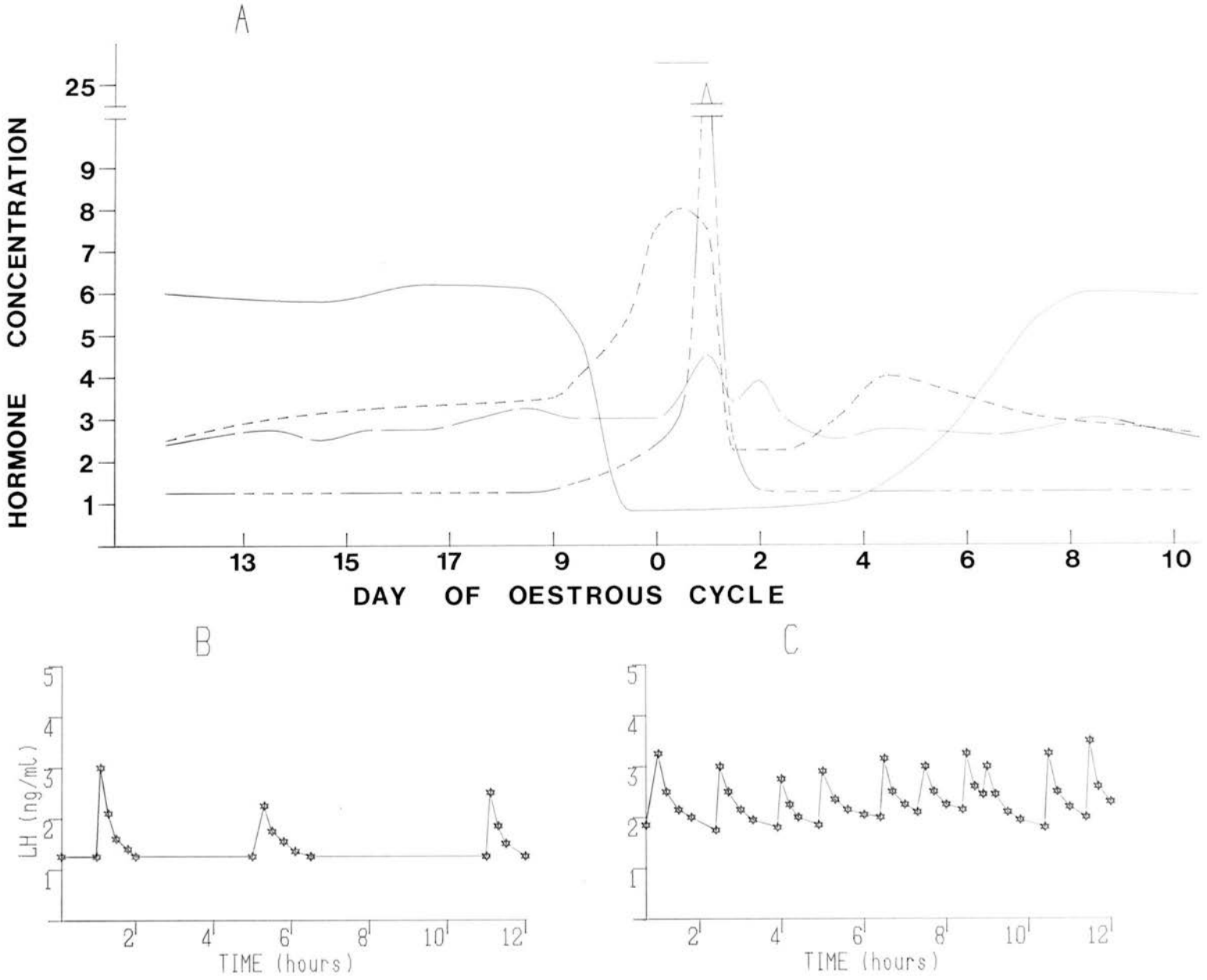
An action of the pituitary gland upon the ovary was discovered when extracts from sheep pituitary glands were given to rats & rabbits, and exhibited both a stimulating and a luteinising effect on follicles (Evans, 1924, cited by Parkes, 1929; Zondek & Aschheim, 1927, cited by Parkes, 1929; Fevold, Hisaw, Hellbaum & Hertz, 1933; Evans, Simpson, Tolksdorf & Jensen, 1939). The active principles were named follicle-stimulating hormone and luteinising hormone, respectively, in the female. Initially, LH activity was measured in the ovarian ascorbic acid depletion bioassay of Parlow (1961), and FSH activity was estimated by measuring the ovarian weight increase induced by sample preparations augmented with known amounts of LH activity (Steelman & Pohley, 1953).

The purification of these two hormones proceeded in a variety of species, the bovine hormones being isolated in 1962 (Reichert) and 1965 (Reichert & Jiang) for LH & FSH respectively, and were found to be glycoproteins with a molecular weight of approximately 30kDa.

FIGURE 1.1

A. Schematic representation of the endocrine patterns throughout the oestrous cycle of the cow. Figure shows representative concentrations of circulating LH (——·····; ng/ml), FSH (—— —— ——; x10ng/ml), progesterone (————; ng/ml) and oestradiol (-------; pg/ml); the red bar indicates behavioural oestrus.

The pulsatile nature of LH release is illustrated in the luteal phase (B) and in the follicular phase (C).


A
HORMONE CONCENTRATION
25
9
8
7
6
5
4
3
2
1
13
15
17
9
0
2
4
6
8
10
DAY OF OESTROUS CYCLE
B
LH (ng/mL)
5
4
3
2
1
2
4
6
8
10
12
TIME (hours)
C
5
4
3
2
1
2
4
6
8
10
12
TIME (hours)

Whilst purifying LH, it was reported that acid conditions encouraged the dissociation of ovine LH (Li & Starman, 1964), which suggested the occurrence of subunits. It is now known that both gonadotrophins exist as two subunits; these subunits were nominated α- and β-units, and found to have molecular weights of approximately 16kDa. The gene coding for the bovine α-subunit has been characterised and sequenced (Goodwin, Moncman, Rottman & Nilson, 1983). The α-subunit is common to both gonadotrophins, whilst the β-subunit is hormone-specific (reviewed by Butt, 1975).

1.1.2.1 Actions of the Gonadotrophins

The general stimulating effects of the gonadotrophins on the ovary has been acknowledged for some time (Fevold et al., 1933; Evans et al., 1939; Casida, Meyer, McShan & Wisnicky, 1943), and early use was made of pregnant mares' serum gonadotrophin (PMSG; see Butt, 1975) to increase the number of preovulatory follicles and thus ovulation rate in cattle (Folley & Malpress, 1945). LH has been shown to regulate the growth of preovulatory follicles (rat: Richards, Jonassen & Kersey, 1980; ewe: McNatty, Gibb, Dobson & Thurley, 1981; human: Mais, Kazer, Cetel, Rivier, Vale & Yen, 1986), induce ovulation (human: Townsend, Brown, Johnstone, Adey, Evans & Taft, 1966; Brown, Evans, Adey, Taft & Townsend, 1969; Messinis, Templeton & Baird, 1986; rat: Shaykh, LeMaire, Papkoff, Curry, Sogn & Koos, 1985; cow: DeSilva, Tjoelker, Chang & Reeves, 1986; sheep: Wallace, McNeilly & Baird, 1986), and stimulate the growth of the corpus luteum (cattle: Donaldson, Hansel & van Vleck, 1965; Hansel & Seifart, 1967; Snook, Brunner, Saatman & Hansel,

1969; Hansel & Snook, 1970; Hoffman, Schams, Bopp, Ender, Gimenez & Karg, 1974; goats: Mohini, & Chapekar, 1983; primates: Mais et al., 1986), whilst during the preovulatory surge it appears to exert an inhibitory influence on follicular oestradiol production (cow: Dieleman, Bevers, Poortman & van Tol, 1983).

More recent studies have helped to define the precise manner in which LH & FSH act upon the ovary. Receptors for LH or human chorionic gonadotrophin (hCG; possesses similar biological properties as LH, see Butt, 1975) have been identified on thecal and luteal cells in several species (rat: Rajaniemi & Vanha-Perttula, 1972; Richards, Ireland, Rao, Bernath, Midgley & Reichert, 1976; pig: Ziecik, Shaw & Flint, 1980; cow: Merz, Hauser & England, 1981; Henderson, Kieboom, McNatty, Lun & Heath, 1984a), and in the granulosa cells of large, preovulatory follicles (pig: Channing & Kammerman, 1973, 1974; rat: Nimrod, Bedrak & Lamprecht, 1977; sheep: Webb & England, 1982a, b). FSH receptors have been located exclusively on granulosa cells (pig: Kammerman, Canfield, Kolena & Channing, 1972; Channing & Kammerman, 1974; sheep: Carson, Findlay, Burger & Trounson, 1979; cow: Merz et al., 1981).

The major effect of LH on the theca cell is to stimulate the synthesis of androgens (rat: Armstrong & Papkoff, 1976; cow: Henderson et al., 1984a), whereas FSH stimulates oestradiol production from the granulosa cell (rat: Armstrong & Papkoff, 1976; Erickson & Hsueh, 1978a; sheep: McNatty, Hudson, Gibb, Ball, Henderson, Heath, Lun & Kieboom, 1985a); these effects are mediated by cAMP activity (rat: Nordenstrom, Rosberg & Roos, 1985; sheep: Weiss, Seamark, McIntosh & Moor, 1976) with some calcium and calmodulin involvement (rat: Eckstein, Eshel, Eli, Ayalon & Naor, 1986), and the synthesis of DNA (hamster: Roy & Greenwald, 1986).

FSH has also been demonstrated to stimulate the production of LH and FSH receptors on the granulosa cell membrane (rat: Zeleznik, Midgley & Reichert, 1974; sheep: Weiss, Armstrong, McIntosh & Seamark, 1978).

In addition, some actions of the gonadotrophins may operate through neural circuits within the ovary, and involve intra-ovarian catecholamines (see Spicer, 1986). However, these basic actions of the gonadotrophins are dependent on the stage of the oestrous cycle, and on the age and health of the follicle. There is a complex interaction between the gonadotrophins and ovarian steroids at the ovarian cell level, and these have been reviewed by many authors (Richards & Midgley, 1976; Richards, Rao & Ireland, 1978; Henderson, 1979; Leung & Armstrong, 1980; Baird & McNeilly, 1981; McNatty, 1981; Hillier, 1981; Baird, 1983; Spicer & Echternkamp, 1986); these aspects will be considered in more detail in section 1.1.3.3. At this point it is more relevant to consider the factors which control the release of LH and of FSH.

1.1.2.2 Central Control of Gonadotrophin Secretion.

It has been known for some time that the secretion of the gonadotrophins is under the direct control of the higher centres of the brain. Specific hypothalamic lesions (Clegg & Ganong, 1960; Schneider, Crighton & McCann, 1969) or pharmacological agents that block neurotransmission (Hansel & Trimberger, 1951) were shown to prevent ovulation in several species, which suggested a neural control located within the hypothalamus. Hypothalamic extracts from a variety of species were shown to be potent stimulators of LH

secretion (McCann, Taleisnik & Friedman, 1960; McCann, 1962; Schally & Bowers, 1964) and ovarian activity (Domanski & Kochman, 1968); the humoral agent extracted from the hypothalamus was called, not surprisingly, gonadotrophin releasing hormone (GnRH). These results confirmed the earlier hypothesis that this hormone was released from the hypothalamus as a result of a neural stimulus, and then transported to the pituitary gland by a discrete blood supply (Green & Harris, 1946).

Gonadotrophin releasing hormone was first purified by Matsuo, Arimura, Nair & Schally (1971a) in the pig and the resultant decapeptide synthesised (Matsuo, Baba, Nair, Arimura & Schally, 1971b). More recently, the DNA encoding the GnRH precursor molecule has been characterised (Seeburg & Adelman, 1984). Pure and synthetic hormone preparations enabled the development of radioimmunoassay techniques and detailed biological studies. Radiolabelled GnRH was used to identify the GnRH secreting neurones in rodents, monkeys, cattle and sheep, and these were found to be distributed in the medial preoptic and septal areas of the brain, and to converge in the median eminence and pituitary stalk (Kizer, Palkovits, Tappaz, Kebabian & Brownstein, 1976; Estes, Padmanabhan & Convey, 1977). Endopeptidases which cleave GnRH have also been reported to occur in the pituitary stalk (cow: Wilk & Orlowski, 1980; rat: Leblanc, L'Heritier, Kordon, Horsthemke, Bauer, Wattiaux-de Coninck, Dubois & Wattiaux, 1984). GnRH has been measured by radioimmunoassay in the hypothalamus and portal blood of several species, although its presence in the peripheral circulation has not been fully characterised (Crighton Foster, Holland & Jeffcoate, 1973; Caraty, De Reviers, Pelletier & Dubois, 1980).

Biological studies showed that GnRH preparations readily induced

detectable releases of LH & FSH in sheep (Lincoln, 1979), cattle (Kittok, Britt & Convey, 1973; Kesner & Convey, 1982), and horses (Alexander & Irvine, 1986), with a greater response being obtained just before the preovulatory gonadotrophin surge (sheep: Foster & Crighton, 1976; cow: Convey, Beal, Seguin, Tannen & Lin, 1976; Leung, Padmanabhan, Convey, Short & Staigmiller, 1984; horse: Alexander & Irvine, 1986). Conversly, a reduction in LH pulsatile secretion was reported in sheep immunised against GnRH (Clarke, Fraser & McNeilly, 1978; Lincoln & Fraser, 1979; Fraser & McNeilly, 1983; Caraty, Martin & Montgomery, 1984) and in rats treated with a GnRH antagonist (Grady, Shin, Charlesworth, Cohen-Becker, Smith, Rivier, Rivier, Vale & Schwartz, 1985), and thus follicular growth and development (rat: Debeljuk & Schally, 1986; sheep: McNeilly, Jonassen & Fraser, 1986). These studies raised several important questions about the action of GnRH. Firstly, alterations of GnRH secretion altered most markedly LH pulse frequency, suggesting that GnRH itself may be released in a pulsatile manner; secondly, discrepancies arose in the effects of GnRH treatments upon LH and FSH release, which suggested the existence of separate releasing hormones for each gonadotrophin.

Ovariectomised animals show a very regular (within animal) pattern of pulsatile LH release, with a pulse interval of approximately one hour (monkey: Dierschke, Bhattacharya, Atkinson & Knobil, 1970; cow: Rahe, Fleeger & Harms, 1982; sheep: Rawlings, Jeffcoate & Rieger, 1984), which further suggests that GnRH release is also pulsatile. Direct evidence came from measurements of GnRH in the hypophyseal portal blood of rhesus monkeys (Carmel, Araki & Ferin, 1976), where regular pulses were readily detected in ovariectomised and follicular phase monkeys. This implied that the

neurones which stimulated GnRH release fired in unison, and were regulated by a rhythm-generating unit, the 'pulsar' of Martin (1984). Once the technical difficulties of cannulating the hypophyseal portal vessels, whilst maintaining pituitary function had been overcome, it was shown that every LH pulse occurred immediately following a portal GnRH pulse in sheep (Clarke & Cummins, 1982; Levine, Pau, Ramirez & Jackson, 1982), although not every GnRH pulse was followed by a LH pulse.

The biological importance of GnRH pulse release has been investigated by comparison of either pulsatile or continuous GnRH infusion into rats, sheep & cattle. In the luteal phase cow, consecutive bolus injections of GnRH at 2 hour intervals caused consecutive surge-like releases of LH, with the magnitude of each release decreasing with each consecutive injection (Kittok et al., 1973). Continuous infusion of GnRH into ovariectomised ewes was found to cause an initial surge of both gonadotrophins, but concentrations declined to baseline within 12 hours, even though GnRH infusion continued (Nett, Crowder, Moss & Duello, 1981; Crowder, Herring & Nett, 1986). These authors also noted a decrease in the GnRH receptor content of the pituitary glands of the infused sheep, and proposed that constant exposure of the pituitary gland to GnRH caused 'down regulation' of the receptors, and thus desensitised the gland. Similar effects have been observed in the anoestrous ewe (Amundson & Wheaton, 1979), although the cause of decreased LH output was given as depletion of pituitary LH content; both systems may operate in vivo.

Dispersed sheep pituitary cell systems were used by McIntosh & McIntosh (1983, 1985, 1986) to examine the effects of a variety of GnRH signals on LH output. Maximal LH output was achieved by

stimulating the cells with long, infrequent pulses of GnRH. Very rapid GnRH pulsing, or constant infusion, caused down regulation of the pituitary cells. These studies were extended using ovariectomised ewes with hypothalamo-pituitary disconnections (Clarke, Cummins, Findlay, Burman & Doughton, 1984a), and a direct relationship between exogenous GnRH pulse amplitude and endogenous LH pulse amplitude was found, in addition to the relationship between GnRH pulse frequency and LH pulse frequency; a similar relationship between endogenous GnRH pulse amplitude and LH pulse amplitude had been reported previously (Levine et al., 1982).

Alternatively, it has been suggested that GnRH pulse frequency can account for both LH pulse frequency and pulse amplitude in ovariectomised ewes with hypothalamo-pituitary disconnections, and given defined pulses of GnRH (Clarke & Cummins, 1985). This theory is based on the existence of two or more pools of pituitary LH (Yen & Lein, 1976; Stelmasiak & Cumming, 1977), one of which could be a readily releasable pool. According to Clarke & Cummins (1985), the size of the releasable pool would be regulated by the frequency with which it was emptied, i.e., by GnRH pulse frequency; thus GnRH pulse frequency determines LH pulse amplitude, as a function of the size of the releasable pool of LH. However, this theory neglects the factors which may regulate the entry of LH into the releasable pool. Ovariectomised ewes actively immunised against GnRH were shown to have significantly reduced pituitary contents of immunoreactive LH and FSH (Adams & Adams, 1986), which suggests that GnRH stimulates the entry of LH into at least one pituitary pool.

Clayton (1982) observed that infusion of very low doses of GnRH into rats (as might be expected to occur in vivo between pulses) caused an 'up regulation' of pituitary LH receptors, and primed the

pituitary in readiness for each GnRH pulse. Constant GnRH stimulation has been found to be necessary for pituitary function during the preovulatory LH surge in sheep (Webb, England & Fitzpatrick, 1981), and similar results have been reported for rat hemipituitary preparations (Pickering & Fink, 1979). The primate may differ from the sheep and the rat in its dependence upon GnRH activity at this time (Fraser, McNeilly, Abbott & Steiner, 1986a). Disconnection of the pituitary from the hypothalamus in sheep led to decreased pituitary responsiveness to exogenous GnRH, implying a lack of GnRH receptors &/or decrease of LH synthesis (Clarke et al., 1984a). A similar decrease in pituitary GnRH receptors was observed in rats actively immunised against GnRH, giving further evidence that GnRH is required for the maintenance of its own receptors (Popkin & Fraser, 1985). In summary, it appears from the literature that a constant low level of GnRH secretion might be required to maintain a functional pituitary gland, but that LH pulsatile release is effected by discrete pulses of GnRH.

The question of two gonadotrophin releasing hormones remains controversial. GnRH administration has been reported to cause similar patterns of LH & FSH release from sheep pituitary cells in culture (McIntosh & McIntosh, 1986) and in vivo (Nett et al., 1981), and antagonists administered to rat pituitary cells caused similar patterns of LH and FSH suppression (Grady et al., 1985). However, most of the available evidence suggests the existence of a differential control of LH and of FSH secretion. GnRH agonists preferentially stimulated LH release over FSH release in ovariectomised rats (Berardo & DePaolo, 1986; de Greef, de Koning, Tijssen & Karels, 1987) and hamsters (Chappel, Miller & Hyland, 1984a). The effect of a GnRH antagonist on LH secretion was more

marked than its effects on FSH secretion in ovariectomised rats (Grady et al., 1985; Schwartz, Rivier, Rivier & Vale, 1985) and cyclic monkeys (Fraser, Abbott, Laird, McNeilly, Nestor & Vickery, 1986b). Ovariectomised rats given phenobarbital were found to have decreased mean concentrations and pulse frequency of both LH and FSH, but only a decrease in the pulse amplitude of LH (DePaolo, 1985). These studies also indicated that phenobarbital increased the LH-response of the pituitary gland to GnRH, but not the FSH-response. The administration of GnRH antisera (Narayana & Dobson, 1979) or antagonist (Condon, Heber, Stewart, Sawyer & Whitmoyer, 1984) blocked or delayed the preovulatory gonadotrophin surge in intact rats and sheep , but did not affect the secondary FSH surge. Constant infusion of GnRH to ovariectomised ewes with complete hypothalamo-pituitary disconnections caused the cessation of LH release, whilst FSH secretion continued, although at lower concentrations than those of ewes given GnRH pulses (Clarke, Burman, Doughton & Cummins, 1986a).

In some studies, active (Clarke et al., 1978) and passive (Lincoln & Fraser, 1979) immunisation of sheep against GnRH blocked pulsatile LH release without affecting FSH secretion, whereas in others, FSH secretion was also decreased (Adams & Adams, 1986). Fraser & McNeilly (1983) passively immunised follicular phase ewes and blocked LH release & found an increase in FSH secretion, although McNeilly et al., (1986) actively immunised seasonally anoestrous ewes and found decreased circulating concentrations of LH and FSH, but not prolactin. Active immunisation of mares against GnRH decreased circulating concentrations of both LH and FSH, but FSH secretion was suppressed to a lesser extent than that of LH (Garza, Thompson, French, Wiest, St.George, Ashley, Jones, Mitchell

& McNeill, 1986). Further to these studies, several authors have reported that several molecular forms of FSH may exist in some species (rats: Foulds & Robertson, 1983; Ulloa-Aguirre, Torra, Dominguez, Scherpbier & Larrea, 1985; Chappel & Ramaley, 1985; hamster: Chappel, 1981; monkey: Chappel, Bethea & Spies, 1984b), and suggest that GnRH may alter the ratios of the FSH molecules secreted.

Tenuous evidence for the existence of a specific FSH-releasing hormone came from the studies of Currie, Johansson, Folkers & Bowers (1973) and Johansson, Currie, Folkers & Bowers (1973), who compared the biopotency of various hypothalamic extracts with purified GnRH, and was strengthened by the discovery of FSH-releasing activity in hypothalamic extracts which were essentially free of GnRH activity (Bowers, Currie, Johansson & Folkers, 1973; Mizunuma, Samson, Lumpkin & McCann, 1983). However, this FSH-releasing agent was never isolated, and other authors have been unable to isolate a FSH-releasing hormone from purified GnRH preparations (Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk & White, 1971a; Schally, Arimura, Kastin, Matsuo, Baba, Redding, Nair, Debeljuk & White, 1971b; Schally, Arimura, Redding, Debeljuk, Carter, Dupont & Vilchez-Martinez, 1976). Analysis of the GnRH precursor molecule has indicated the presence of a 56 amino acid peptide (Seeburg & Adelman, 1984), whose physiological function is uncertain, but which stimulated FSH secretion and inhibited prolactin secretion from rat pituitary cells in vitro (Nikolics, Mason, Szonyi, Ramachandran & Seeburg, 1985). Taken together, these results indicate that FSH secretion is stimulated by the releasing hormone common with LH, and that there is also a GnRH-independent control, possibly mediated by ovarian factors (see section 1.1.3.3).

Studies on the mechanism of action of GnRH at the pituitary cell have indicated that GnRH binds to a single class of high affinity receptors in the pituitary cell plasma membrane (Wagner, Adams & Nett, 1979) and stimulates the synthesis of both subunits of LH (Starzec, Counis & Jutisz, 1986); GnRH was found to be essential for the maintenance of pituitary mRNA for both sub-units of LH in sheep (Hamernik, Crowder, Nilson & Nett, 1986). The second messenger for GnRH has been found to be calcium, with the involvement of the calcium binding protein, calmodulin (Drouva, Laplante & Kordon, 1985); the major cyclic nucleotides do not appear to act as second messengers in this system (Catt, Loumaye, Wynn, Suarez-Quian, Kiesel, Iwashita, Hirota, Morgan & Chang, 1984). Increased phospholipid turnover has also been observed in pituitary cells upon GnRH stimulation (Naor & Catt, 1981), and has been implicated in hormone action (Nishizuka, 1984). It has been suggested that the increased phospholipid turnover activates protein kinase C, and, together with activated calmodulin, stimulates gonadotrophin synthesis and release (reviewed by Conn, 1986).

GnRH is not the only biologically active molecule to be found in the median eminence (Kizer et al., 1976). The turnover of serotonin was found to be higher at oestrus than at other times of the cycle in sheep (Wheaton, Martin, Swanson & Stormshak, 1972), suggesting an involvement in the gonadotrophin surge. Subsequent to this study, catecholamines have been reported to influence gonadotrophin secretion. Adrenergic blocking agents were found to reduce basal LH release in ovariectomised ewes (Jackson, 1977), and to block pulsatile LH release in ovariectomised rats (Weick, 1978); a similar treatment decreased LH, but not FSH, in ovariectomised hamsters (Chappel et al., 1984a). A low level of noradrenaline

decreased LH pulse frequency when infused into the medial preoptic area of ovariectomised, conscious rats (Leipheimer & Gallo, 1985; Condon, Handa, Gorski, Sawyer & Whitmoyer, 1986). Noradrenaline injections have been reported to have no effect on gonadotrophin secretion in the human female (Paradisi, Venturoli, Spada, Porcu, Magrini, Giambiasi, Capelli & Flamigni, 1986). In contrast, dopamine antagonists have been reported to increase LH, and, to a lesser extent, FSH secretion in ovariectomised hamsters (Chappel et al., 1984a), and dopamine receptor stimulants markedly decreased pulsatile LH release in ovariectomised rats (Drouva & Gallo, 1976). In general, it is considered that GnRH secretion is controlled by a fine balance between dopaminergic and adrenergic neural input to the hypothalamus.

There is also evidence which suggests that the endogenous opioid peptides control GnRH secretion. The presence of β-endorphin has been reported in the rat pituitary gland (Guillemin, Vargo, Rossier, Minick, Ling, Rivier, Vale & Bloom, 1977) and in monkey hypophyseal portal blood (Wehrenberg, Wardlaw, Frantz & Ferin, 1982). The incubation of rat hypothalamus slices with β-endorphin, Met- or Leu-enkephalin, or morphine inhibited potassium ion-induced GnRH release, which could be reversed by the addition of the opiate antagonist naloxone (Drouva, Epelbaum, Tapia-Arancibia, Laplante & Kordon, 1981). Other agents are also effective in altering gonadotrophin release. Neurotensin has been shown to increase LH secretion when injected into the preoptic area of anaesthetised, ovariectomised rats, and receptors for neurotensin have been reported in the preoptic area (Ferris, Pan, Singer, Boyd, Carraway & Leeman, 1984), and a pancreatic polypeptide has been reported to decrease LH secretion in ovariectomised rats (Kalra & Crowley,

1984). Very recently, a 12 kDa protein has been isolated from the rat hypothalamus, which inhibited GnRH-stimulated LH release (Hwan & Freeman, 1987).

Some authors have postulated the existence of a short-loop feedback from the pituitary gland to the hypothalamus, and an ultrashort-loop feedback from within the hypothalamus, to control gonadotrophin secretion. Evidence has been reported for the control of growth hormone release by somatostatin in rats (McCann, 1980), for the control of FSH release by FSH in the rabbit (Patritti Laborde, Wolfsen & Odell, 1981) and rat (Ojeda & Ramirez, 1969), for the control of LH by LH (Ojeda & Ramirez, 1969), and GnRH by GnRH (DePaolo, King & Carrillo, 1987). Prolactin has also been implicated in the control of gonadotrophin secretion, but this involvement is probably limited to periods of anoestrus rather than during the oestrous cycle (McNeilly, 1980; Forrest, Fleeger, Long, Sorensen & Harms, 1980).

Confirmation of the roles of these compounds and mechanisms must await further study, especially for the larger domestic species, but indicates the complexity of the central control of gonadotrophin secretion, and provides several planes at which central control mechanisms can be modified by other physiological influences, for instance, hormonal messages from the ovaries. This latter is of particular interest in the light of findings of specific steroid concentrating neurones within the brain, at least in the rat (see Kalra & Kalra, 1984).

1.1.3 OVARIAN STEROID SECRETION

1.1.3.1 Structure and Synthesis

The biochemical pathways from cholesterol to the ovarian steroid hormones is well established (see Butt, 1976). There are two major routes from cholesterol to testosterone (Figure 1.2), named the delta-4 and delta-5 pathways. In the bovine ovary, the delta-5 pathway appears to be the predominant route (Lacroix, Eechaute & Leusen, 1974; Fortune, 1986).

Whilst both granulosa and theca cells are able to secrete the major ovarian steroids, there is good evidence that these two cell types interact in the overall secretion of the hormones. The earliest evidence came from the studies of Falck (1959) with autotransplants of granulosa cells, theca cells, or both, into the anterior chamber of the eyes of rats. These results formed the basis of the 'two-cell' theory of oestrogen biosynthesis, that the thecal tissue secretes androgens, which are exported to the granulosa cells for conversion into oestrogens.

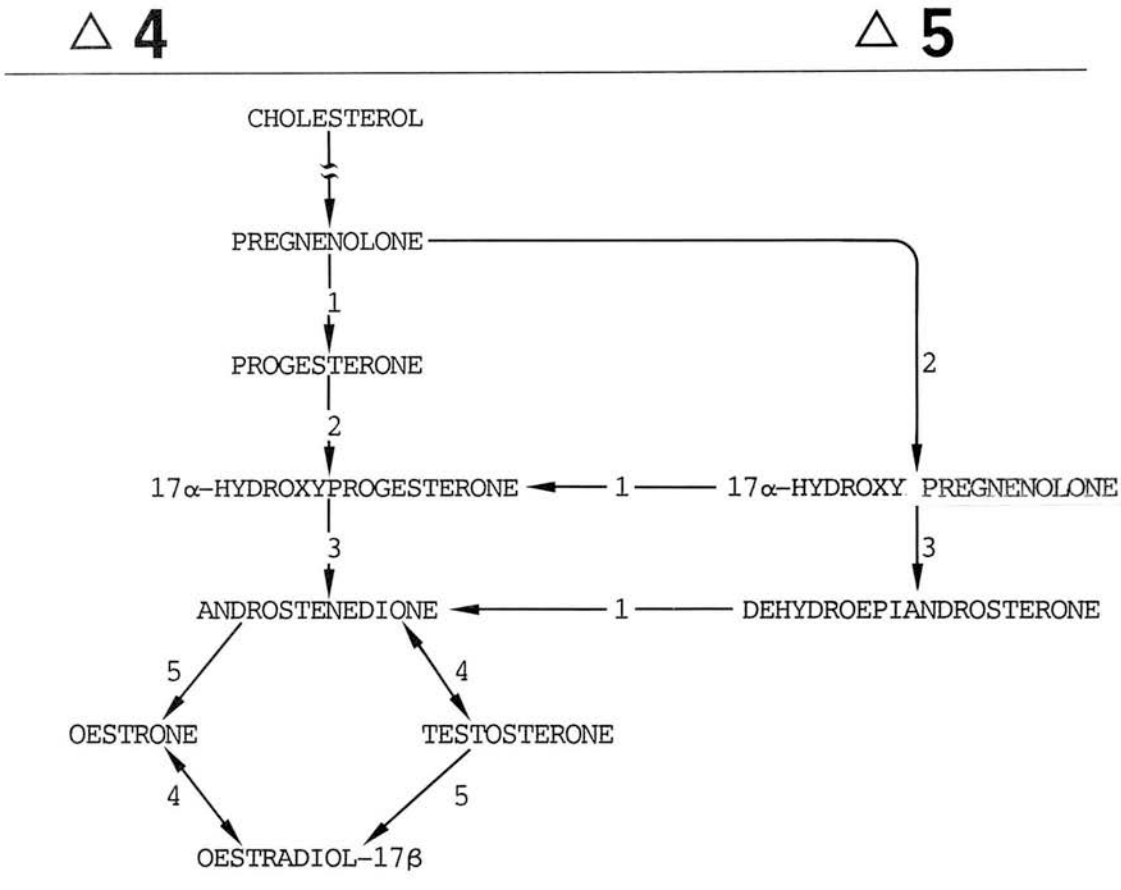
In support of the two-cell theory, granulosa cell production of androgens is considered to be negligible in the rat (Fortune & Armstrong, 1977; Liu & Hsueh, 1986), pig (Evans, Dobias, King & Armstrong, 1981), horse (Channing, 1969a), human (Channing, 1969b), sheep (Moor, 1977; Moor, Hay & Seamark, 1975) and cow (Fortune, 1986), and, similarly, thecal production of oestrogen is considered to be low in the rat (Fortune & Armstrong, 1978), pig (Tsang, Leung & Armstrong, 1979), sheep (Moor, 1977) and cow (Lacroix et al.,

1974). Furthermore, incubation of both tissue types together, or of granulosa cells in the presence of androgen precursor, resulted in a greater oestrogen production than that by granulosa cells alone (human: Ryan, Petro & Kaiser, 1968; monkey: Channing, 1980; sheep: Moor, 1977; hamster: Makris & Ryan, 1975; horse: Ryan & Short, 1965; rat: Oakey & Stitch, 1967; Liu & Hsueh, 1986; cow: Lacroix et al., 1974).

However, the theca cell has been demonstrated to possess at least a limited capability to secrete oestrogen in the human (Channing, 1969b), horse (Younglai & Short, 1970), monkey (Channing & Coudert, 1976) and pig (Stoklosowa, Bahr & Gregoraszczuk, 1978; Tonetta, DeVinna & diZerega, 1986). One important question needs to be addressed, and that is, is steroid interaction within the ovary restricted to precursor exchange, or do steroids have regulatory actions. Recent evidence suggests the latter, and that these actions are integral to follicular development.

FIGURE 1.2

Pathways involved in the synthesis of the major ovarian steroid hormones.



Major enzymes;

1, 3β-hydroxysteroid dehydrogenase 2, 17α-hydroxylase

3, C17-C20 lyase 4, 17β-hydroxysteroid dehydrogenase

5, Aromatase enzyme system

Compiled from Butt (1976) and Feder (1981).

1.1.3.2 Intra-ovarian Action of Steroids

Oestradiol has been shown to have several actions within the ovary, at least in the rat, which include the stimulation of granulosa cell proliferation (Goldenberg, Vaitukaitis & Ross, 1972) and the enhancement of FSH binding to granulosa cells (Richards, 1975; Richards & Midgely, 1976). Thus the small, immature follicle, which possesses receptors for FSH on the granulosa cells (mice: Eshkol & Lunenfeld, 1972; pig: Channing & Kammerman, 1974; sheep: Carson et al., 1979), can synthesise oestradiol, which acts within the follicle to promote further FSH-induced oestradiol secretion and follicular growth.

Since granulosa cells synthesise little androgen, a continued output of oestradiol is dependent upon the activity of the theca cell. Small follicles have been reported to possess LH receptors on the theca cell (rat: Rajaniemi & Vanha-Perttula, 1972; Lee & Ryan, 1973; pig: Ziecik, Shaw & Flint, 1980), although the responsiveness of the small follicle to LH was reported to be lower than that of larger follicles in the pig (Channing & Kammerman, 1974).

Appropriate LH stimuli have been shown to increase the secretion of thecal androgens (see Leung & Armstrong, 1980), which could act not only as precursors for oestradiol synthesis (Schomberg, Stouffer & Tyrey, 1976), but also as activators of further aromatase enzyme activity (rat: Daniel & Armstrong, 1980; Hillier & DeZwart, 1981). An increase in follicular oestradiol secretion has been demonstrated to stimulate FSH-induced aromatase enzyme activity (Adashi & Hsueh, 1982; Zhuang, Adashi & Hsueh, 1982). Increased intra-follicular concentrations of oestradiol could allow an interaction between FSH

and oestradiol, which was reported to stimulate the proliferation of receptors for LH on the theca layer, and ultimately on the granulosa cells (pig: Channing & Kammerman, 1974) as the follicle enters the rapid growth phase up to pre-ovulatory size. The ability of FSH to induce the formation of receptors for LH on the granulosa cell was recently shown to be enhanced by transforming growth factor-β in rats (Knecht, Peng & Catt, 1986).

Some steroids are reported to have suppressive actions within the ovary. Progesterone inhibited the induction of aromatase activity by FSH in rats (Fortune & Vincent, 1983), reduced basal oestradiol secretion by the porcine granulosa cell (Chan & Tan, 1986) and was suggested to decrease ovarian 17α-hydroxylase and C17-20 lyase activities in the hamster (Johnson, 1987). There is other, indirect, evidence for a suppressive action of progesterone upon oestradiol secretion in the cow. During the luteal phase of the cycle, pulses of ovarian oestradiol were released approximately 60 min after a pituitary LH pulse, whereas during the follicular phase this time lag was reduced to 20 min (see section 1.1.1). In another study, an increase in plasma oestradiol concentration was observed after luteolysis in anaesthetised cattle, despite the absence of a rise in mean LH concentrations (Fogwell, Weems, Lewis, Butcher & Inskeep, 1978).

Androgens were shown to stimulate progesterone synthesis in the granulosa cells of pigs (Haney & Schomberg, 1978) and rats (Nimrod & Pearlsman, 1984) and to stimulate pregnenolone secretion by bovine granulosa cells (Fortune, 1986). Pregnenolone secretion has also been reported in the LH stimulated theca cell of the cow (Shemesh & Ailenberg, 1977). High intrafollicular concentrations of androgens and progesterone (McNatty et al., 1984b), and low intrafollicular

concentrations of oestrogen (Merz et al., 1981; Tsonis, Carson & Findlay, 1984) are indicators of follicular atresia.

Oestradiol inhibited progesterone synthesis in the pig (Thanki & Channing, 1978; Haney & Schomberg, 1978) and cow (Williams & Marsh, 1978; Fortune & Hansel, 1979; Henderson, McNatty, Smith, Gibb, O'Keeffe, Lun, Heath & Prisk, 1987), and inhibited androgen secretion in rats (Leung & Armstrong, 1979; Magoffin & Erickson, 1981). Thus the ability of a follicle to maintain a high potential to secrete oestradiol is essential for its maturation into a preovulatory follicle. Indeed, it has been shown that treatment with FSH or PMSG decreases the proportion of atretic follicles and increases the viability of the follicles in sheep (McNatty et al., 1985a) and cattle (Monniaux, Mariana & Gibson, 1984).

In addition to the intra-ovarian steroid exchanges described above, Fortune (1986) suggested that pregnenolone produced by granulosa cells could be used by the theca cells of healthy bovine follicles as a substrate for androstenedione production (and thus also oestradiol synthesis), and that intrafollicular oestradiol inhibits 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, thus reducing progesterone synthesis in favour of further pregnenolone secretion. The healthy follicle thus utilises several pathways to accumulate high concentrations of oestradiol. The factors which determine whether or not a follicle loses this ability for sustained oestrogen synthesis, and thus becomes atretic, are unclear. It is known that gonadotrophic support is crucial for follicular development (see above), and that the ovary contributes to the control of gonadotrophin secretion.

1.1.3.3 Ovarian Steroid Control of Gonadotrophin Secretion

It has long been recognised that the gonad plays an essential role in the control of reproductive function. A direct relationship between the testis and the pituitary gland was noted by Mottram & Cramer in 1923, and ovariectomy has been shown to lead to degeneration of the uterus, oviducts and mammary glands as well as diminishing libido in several species, and causing hypertrophy of the pituitary gland (cited by Parkes, 1929; Martins & Rocha, 1931). Conversely, the administration of ovarian extracts was found to enhance libido and the growth of ovarian structures; these early results were inconsistent (probably owing to the use of water-based extracts, and thus of variable steroid content) and without true 'control' subjects (Brown-Sequard, 1889; Variot, 1889; Marshall & Jolly, 1905). It was not long before lipid and alcohol extracts were prepared from the whole ovary and from isolated follicles, which were potent for inducing behavioural oestrus in laboratory animals, and thus termed oestrogenic (Fellner, cited by Parkes, 1929). Similarly, progesterone was discovered in extracts prepared from corpora lutea; these extracts inhibited oestrus (Papanicolaou, 1926; Hammond, 1927; Parkes & Bellerby, 1927) and caused hypertrophy of the uterus (Iscovesco, 1912a, b).

As assays for plasma gonadotrophins became available, it was shown that the pituitary hypertrophy seen in ovariectomised animals was associated with elevated plasma gonadotrophin concentrations (cow: Hobson & Hansel, 1972a, b; Short, Howland, Randel, Christensen & Bellows, 1973; Convey, Beck, Neitzel, Bostwick & Hafs, 1977; Forrest et al., 1980; Schallenberger & Peterson, 1982; Butler, Katz, Arriola, Milvae & Foote, 1983; sheep: Niswender,

Roche, Foster & Midgley, 1968; Roche, Foster, Karsch & Dziuk, 1970; Davis & Borger, 1974; monkey: Atkinson, Bhattacharya, Monroe, Dierschke & Knobil, 1970; rat: Meyer & Hertz, 1937; Tapper, Naftolin & Brown-Grant, 1972; Ramirez & Sawyer, 1974; mouse: Bronson, 1981).

1.1.3.3.1 Control of LH Secretion

Oestradiol has been shown to cause a long term decrease in mean LH concentrations in ovariectomised rodents (Diekman & Malven, 1973; Bronson, 1981; Clayton & Catt, 1981; Weick & Noh, 1984; Goodman & Daniel, 1985), primates (Karsch, Dierschke, Weick, Yamaji, Hotchkiss & Knobil, 1973), sheep (Goodman, Legan, Ryan, Foster & Karsch, 1980; Moss, Crowder & Nett, 1981; Rawlings et al., 1984) and cattle (Beck, Smith, Seguin & Convey, 1976; Hinshelwood, Dierschke & Hauser, 1986). In the short term, oestradiol administration induced LH surges, with or without a preceding decrease in mean concentrations, in the rodent (Attardi & Happe, 1986; Weiland, Barraclough & Catt, 1986), ewe (Bolt, Kelley & Hawk, 1971; Jackson, 1977; Fraser, Clarke & McNeilly, 1981; Clarke & Cummins, 1984; Nett, Crowder & Wise, 1984; Emons, Schuppe, Peter, Brack & Ball, 1986) and cow (Hobson & Hansel, 1972a, b; Short et al., 1973; Beck & Convey, 1977; Rajamahendran, Lague & Baker, 1979; Short, Randel, Staigmiller & Bellows, 1979; Butler et al., 1983; Schoenemann, Humphrey, Crowder, Nett & Reeves, 1985) and in pituitary cell cultures (rodent: Drouin & Labrie, 1981; cow: Padmanabhan, Kesner & Convey, 1978; Padmanabhan & Convey, 1981). The induction of behavioural oestrus in cattle with injection of oestradiol is well

documented (Rajamahendran et al., 1979; Katz, Oltenacu & Foote, 1980; Nessan & King, 1981). Oestradiol is hence able to exert both negative and positive 'feedback' effects on LH secretion.

A closer examination of the negative feedback actions of oestradiol indicated that LH pulse amplitude is decreased without an effect on pulse frequency in the rat (Goodman & Daniel, 1985; Leipheimer, Bona-Gallo & Gallo, 1986) and ewe (Goodman & Karsch, 1980; Karsch, 1984; Clarke & Cummins, 1984; Rawlings et al., 1984), but pulse frequency was abolished or significantly reduced in other studies (rat: Sylvester, Van Vugt, Aylesworth, Hanson & Meites, 1982; Weick & Noh, 1984; Leipheimer, Bona-Gallo & Gallo, 1985). Further, oestradiol was shown to prevent the post-castration rise in pituitary GnRH receptor numbers in the rat (Clayton & Catt, 1981), but increased receptor numbers in ewes (Moss et al., 1981), and decreased pituitary LH content in ovariectomised ewes without affecting hypothalamic GnRH content (Moss et al., 1981). The inhibition of LH pulse secretion in ovariectomised ewes was not associated with an effect of the oestradiol on hypophyseal portal GnRH pulses (Clarke, 1984). Oestradiol has been reported to decrease pituitary mRNA for the gonadotrophin α-subunit in sheep (Hall & Miller, 1986), and to decrease the pituitary response to GnRH in ovariectomised rats (Turgeon & Waring, 1981) and sheep (Goodman & Karsch, 1980), and in cyclic women (Nagahara, Miyake, Tasaka, Kawamura & Aono, 1984). It has been reported that the negative feedback influence of oestradiol is associated with a significant increase in pituitary nuclear receptors for oestradiol in ovariectomised sheep, whereas the positive effects occur when nuclear oestradiol receptors have returned to baseline (Clarke, Funder & Findlay, 1982). These data imply that oestradiol acts on

the pituitary gland to decrease its sensitivity to GnRH. Recent evidence suggests that oestradiol can modify receptor coupling mechanisms in the pituitary cell (Drouva, Laplante, Lablanc, Bechet, Clauser & Kordon, 1986a), although it is unknown if this is associated with negative or positive feedback.

Other authors have suggested that oestradiol acts at the hypothalamus, or at a higher centre in the brain. Oestradiol inhibited GnRH release into the hypophyseal portal blood of long-term ovariectomised rats (Sarkar & Fink, 1980), and decreased LH pulse frequency in ovariectomised cows (Day et al., 1986), cycling ewes (Wright, Geytenbeek, Clarke & Findlay, 1981) and ewes in seasonal anoestrus (Goodman, Bittman, Foster & Karsch, 1982). Oestradiol has been shown to decrease the secretory activity of GnRH neurones (King, Anthony, Damassa & Elkind-Hirsch, 1987) in ovariectomised rats. Pituitary responsiveness to GnRH was reported to be increased, whilst serum LH concentrations fell in ovariectomised ewes following oestradiol administration (Convey, Kesner, Padmanabhan, Curruthers & Beck, 1981); this suggests an additional inhibitory action of oestradiol other than at the pituitary gland. Recently, the negative feedback effects of oestradiol have been shown to involve catecholaminergic neurones within the hypothalamus in the anoestrous ewe (Meyer and Goodman, 1986) and the ovariectomised, oestrogen-treated rat (Taleisnik & Sawyer, 1986).

Little is known about the control of pulsatile LH release in the cow. Imakawa et al. (1986) reported that oestradiol depressed LH pulse frequency and increased pulse amplitude in ovariectomised heifers.

The positive feedback effects of oestradiol appear to be partly

independent of negative feedback, at least in the primate; in ovariectomised rhesus monkeys, an oestradiol implant reduced circulating LH concentrations to those of the follicular phase of the menstrual cycle, and a bolus oestradiol injection given after implant insertion still elicited a LH surge (Karsch et al., 1973). However, supraphysiological levels of oestradiol have been shown to inhibit the positive actions of oestradiol in rats (Lu, Gilman, Meldrum, Judd & Sawyer, 1981) and sheep (Emons et al., 1986), possibly caused by down-regulation of hypothalamic or pituitary receptors. In the cow, ovariectomy towards the end of oestrus (when oestradiol concentrations are at their highest) did not alter LH secretion (Convey et al., 1977).

The positive feedback influence of oestradiol may also be directed at both hypothalamic and pituitary sites. Administration of oestradiol to bovine pituitary cells has been reported to increase basal LH secretion and the sensitivity of the cells to GnRH (Padmanabhan et al., 1978; Convey et al., 1981; Kesner, Convey & Anderson, 1981; Padmanabhan & Convey, 1981) and to increase the number of pituitary GnRH receptors (Schoenemann et al., 1985). Oestradiol increased the sensitivity of the pituitary gland to GnRH in rats (Drouin & Labrie, 1981), although an effect on pituitary GnRH receptors has been discounted by some authors (rat: Attardi & Happe, 1986; sheep: Wagner et al., 1979). Oestradiol has also been shown to increase LH pulse frequency in sheep; during the late follicular phase, LH pulse frequency was reported to be higher than that of ovariectomised ewes, whilst the pulse amplitude was lower than that of ovariectomised ewes, an observation which was imitated by the administration of oestradiol to ewes ovariectomised in the late luteal phase (Karsch, Foster, Bittman & Goodman, 1983).

Acute administration of oestradiol to hypothalamic slices taken from ovariectomised rats caused an increased GnRH release when stimulated by potassium ions (Drouva, Laplante, Gautron & Kordon, 1984). In addition, oestradiol has been shown to increase mean concentrations and pulse frequency of hypophyseal portal GnRH in acutely ovariectomised rats (Dluzen & Ramirez, 1986), although in ovariectomised sheep, the increase in hypophyseal portal blood concentrations of GnRH did not increase in the same pattern as the LH surge, nor did the GnRH release take the form of a surge (Schillo, Leshin, Kuehl & Jackson, 1985), suggesting a further site(s) of action of oestradiol.

There is evidence that oestradiol acts at centres higher than the hypothalamus, at least in the rat. Catecholamine blockers inhibited oestradiol-induced LH surges in rats (Adler, Johnson, Lynch & Crowley, 1983) and an intra-cerebroventricular injection of noradrenaline stimulated LH release in oestrogen primed, ovariectomised rats (Condon et al., 1986). A similar involvement of the opioid system has been proposed (Sylvester et al., 1982; Kalra & Kalra, 1984). More recently, implants releasing high levels of oestradiol in the ovariectomised rat were shown to cause the induction of oestradiol receptors in higher brain centres, whereas lower oestradiol levels maintained receptors at the hypothalamus and pituitary gland only (Camp & Barraclough, 1985). In the ovariectomised ewe, the positive feedback effects of oestradiol have not been associated with increases in pituitary nuclear receptors for oestradiol (Clarke et al., 1982).

The event(s) which initiates the transition of oestradiol from a negative to a positive feedback agent is unclear. A conspicuous feature of the oestrous cycle is the absence of progesterone during

that phase in which oestradiol normally exerts a positive feedback effect. The oestrus-inhibiting effects of progesterone were established sixty years ago (Papanicolaou, 1926; Hammond, 1927; Parkes & Bellerby, 1927), and the presence of progesterone has been demonstrated to suppress LH surges in ewes (Bolt et al., 1971; Hooley, Baxter, Chamley, Cumming, Jonas & Findlay, 1974; Baird & Scaramuzzi, 1976; Hauger, Karsch & Foster, 1977; Rawlings et al., 1984) and cows (Hansel & Snook, 1970; Hobson & Hansel, 1972a; Rajamahendran et al., 1979; Barnes, Kazmer, Bierley, Richardson & Dickey, 1980; Roche & Ireland, 1981a, b; Kesner, Padmanabhan & Convey, 1982; Schoenemann et al., 1985), and in rat (Drouin & Labrie, 1981) and cow (Padmanabhan & Convey, 1981) pituitary cell cultures. Supraphysiological concentrations of oestradiol have been reported to override the negative effects of progesterone in the cow (Short et al., 1973; Hausler & Malven, 1976).

The feedback effects of progesterone alone are confusing. Progesterone decreased LH pulse amplitude in ovariectomised rats in some reports (Leipheimer, Bona-Gallo & Gallo, 1984), but not in others (Goodman & Daniel, 1985). Results reported for the rat may be of limited physiological importance since the rat does not experience a true luteal phase (see Martin, 1984, for discussion).

In the ovariectomised ewe, progesterone has been reported either to have no effect on LH secretion (Wheaton & Mullett, 1982; Rawlings et al., 1984), or to decrease LH pulse frequency (Goodman & Karsch, 1980; Karsch, 1984; Tamanini, Crowder & Nett, 1986) and mean LH concentrations but not pituitary LH content (Moss et al., 1981). Ewes immunised against progesterone exhibited an increase in LH pulse frequency (Thomas, Oldham, Hoskinson & Scaramuzzi, 1984a). In the ovariectomised cow, progesterone has been shown to cause a

weak suppression of mean LH concentrations (Beck et al., 1976; Roche & Ireland, 1981b); Ireland and Roche (1982b) have reported a negative correlation between progesterone and LH concentrations in progesterone implanted heifers, and Glencross (1987) described the suppression of follicular development by exogenous progesterone, and attributed this to a suppression of LH pulse frequency.

During the oestrous cycle, progesterone has been reported to have no effect on LH secretion in rats (Drouin & Labrie, 1981) or cattle (Padmanabhan & Convey, 1981), although progesterone increased the sensitivity of pro-oestrous rat pituitary cells to GnRH (Turgeon & Waring, 1981). In some species (notably sheep, see Martin, 1984) progesterone pretreatment appears necessary for the full expression of behavioural oestrus.

The disparity of the effects of progesterone on LH secretion is likely to be confounded by the relative exposure of the experimental animals to oestradiol, since there is evidence for an interaction between progesterone and oestradiol in the control of LH secretion.

Treatment with both oestradiol and progesterone has been reported to decrease LH pulse amplitude and frequency to a greater extent than either steroid alone, in ovariectomised rats (Leipheimer et al., 1984; Goodman & Daniel, 1985), ewes (Goodman et al., 1980; Goodman, Pickover & Karsch, 1981b; Martin, Scaramuzzi & Henstridge, 1983; Rawlings et al., 1984) and cattle (Beck et al., 1976). In addition to the above negative feedback effects, progesterone has also been reported to enhance the GnRH-induced LH and FSH surge in oestrogen primed rats (DePaolo & Barraclough, 1979; Drouva et al., 1985), hamsters (Takahashi & Lisk, 1986) and monkeys (Helmond, Simons & Hein, 1980). The physiological significance of this effect is unclear.

The effects of androgens on LH secretion in the female have also received some attention. Androstenedione and testosterone were reported to have no consistent effect on LH concentrations in ovariectomised heifers (Butler et al., 1983), nor reduce the magnitude of the GnRH-induced LH surge in ovariectomised cows (Thompson, Voelkel, Reville-Moroz, Godke & Derrick, 1984). Testosterone was reported to decrease mean LH concentrations in intact pony mares (Reville-Moroz, Thompson, Archibald & Olsen, 1984).

In summary, both oestradiol and progesterone are important negative feedback agents in the ruminant. Oestradiol is the more potent agent, and appears to act predominantly to alter the pituitary response to GnRH, but may also alter GnRH release from the hypothalamus. Progesterone is reported to influence LH pulse frequency by altering GnRH pulse frequency (or the molecular form of GnRH secreted; Drouva, Gautron, Pattou, Laplante & Kordon, 1986b). Together, oestradiol and progesterone reduce both LH pulse frequency and pulse amplitude, thereby reducing circulating LH concentrations in ovariectomised animals to those seen during the oestrous cycle. In the absence of progesterone, but after progesterone priming, oestradiol exerts a positive feedback effect upon the pituitary gland and the hypothalamus to increase pulse frequency and mean concentrations of LH, and to elicit the preovulatory gonadotrophin surge.

1.1.3.3.2 Control of FSH Secretion

In contrast to the ovarian control of LH secretion, little attention has been paid to the feedback control of FSH secretion. There is some evidence for a positive feedback effect of oestradiol in rats (Miller & Wu, 1981), and an augmentation of the pituitary response to GnRH in ovariectomised cattle (Kesner & Convey, 1982), but most of the available evidence suggests a negative feedback action on FSH. Oestradiol has been demonstrated to decrease mean FSH concentrations in ovariectomised monkeys (Chappel et al., 1984b), sheep (Fraser et al., 1981; Goodman et al., 1981b; Moss et al., 1981; Webb, Baxter, Preece, Land & Springbett, 1985a) and cattle (Butler et al., 1983), and in pituitary cell cultures (sheep: Miller, Knight, Grimek & Gorski, 1977; Miller, Alexander, Wu, Huang, Whitfield & Hall, 1983; cow: Miller & Wu, 1981). By contrast, some authors reported that oestradiol had no effect on FSH secretion in ovariectomised cows (Schoenemann et al., 1985) or bovine pituitary cells in culture (Padmanabhan & Convey, 1981). As well as the oestrogen-induced decrease in the pituitary content of mRNA encoding the gonadotrophin α-subunit (Hall & Miller, 1986), Alexander & Miller (1982) and Miller et al. (1983) have reported an oestrogen-induced decrease in the pituitary content of mRNA coding for the FSH β-subunit in sheep. Oestradiol has also been reported to alter the carbohydrate content of the FSH molecule (primates: Wide, 1982; Chappel et al., 1984b; Wide & Hobson, 1986; rats: Ulloa-Aguirre et al., 1985), although the significance of this action is not clear.

Even less is known about the role of the other steroids in the control of FSH secretion. Progesterone had no effect on FSH

secretion in ovariectomised ewes (Moss et al., 1981; Rawlings et al., 1984; Clarke & Cummins, 1984), ovariectomised (Schoenemann et al., 1985) and intact cows (Roche & Ireland, 1981b; Ireland & Roche, 1982b) or in cultured pituitary cells (sheep: Miller et al., 1977; cow: Padmanabhan & Convey, 1981), although other authors have reported a decrease in FSH secretion by ovine pituitary cells (Batra & Miller, 1985). An increase in FSH pulse amplitude was demonstrated in cows after the removal of progesterone-releasing intravaginal devices (PRID; Ireland & Roche, 1982b). Progesterone was reported to increase FSH secretion from rat pituitary cell cultures (Drouin & Labrie, 1981). Testosterone decreased mean FSH concentrations in intact pony mares (Reville-Moroz et al., 1984), but had no consistent effect in ovariectomised cattle (Butler et al., 1983; Thompson et al., 1984).

A combination of oestradiol and progesterone has been reported to be at least as effective as oestradiol alone in reducing FSH secretion in ovariectomised ewes (Goodman et al., 1981b; Moss et al., 1981; Rawlings et al., 1984), but most authors agree that in contrast to LH, FSH concentrations cannot be decreased to those seen during the oestrous cycle. This difference indicates the presence of an additional negative feedback agent from the ovary, and proteins isolated from follicular fluid have received much attention in this respect.

1.1.4 OVARIAN PROTEIN SECRETION

The fluid within antral follicles contain a large range of compounds, most of which are considered to be transudates from the blood (Zachariae & Jensen, 1958; Edwards, 1974). There are also compounds which occur in higher concentrations in follicular fluid than in peripheral blood; these include steroids (see section 1.1.1), prostaglandins (Hunter & Poyser, 1985), certain enzymes (Edwards, 1974), growth factors (Ramasharma, Cabrera & Li, 1986; Hsu & Hammond, 1987), steroid binding proteins (Cook, Hunter & Kelly, 1977; Mahajan & Little, 1978; Petra, Stanczyk, Senear, Namkung, Novy, Ross, Turner & Brown, 1983; Ben-Rafael, Mastroianni, Meloni, Lee & Flickinger, 1986), and proteins of undetermined structure and activity (Moor & Crosby, 1987), which may be species specific (Cabrera, Andino & Mateo de Acosta, 1985).

The gonad secretes a protein(s) which possesses the properties of a hormone. This was first recognised by McCullagh (1932) who argued that the fat soluble steroid hormone obtained from the testis (and termed 'androtin') could not reverse or prevent the castration induced hypertrophy of the pituitary gland, whereas an aqueous testicular extract could prevent pituitary hypertrophy. McCullagh thus proposed a water-soluble gonadal hormone which acted upon the pituitary gland, and called it 'inhibin'. Despite the potential of such a theory, little interest in inhibin was shown before the mid 1970's, when reports demonstrated that water-based extracts of the testis suppressed circulating concentrations of FSH in castrate rams (Lee, Keogh, de Kretser & Hudson, 1974; Setchell & Jacks, 1974; Franchimont, Chari, Hagelstein & Duraiswami, 1975) and that bovine follicular fluid contained a heat-labile substance which suppressed

circulating FSH concentrations in the ovariectomised rat (Hopkinson, Sturm, Daume, Fritze & Hirschhauser, 1975; de Jong & Sharpe, 1976). The definition of inhibin was thus proposed as "a peptidic factor of gonadal origin that specifically or selectively lowers the rate of secretion of FSH" (Franchimont, Verstraelen-Proyard, Hazee-Hagelstein, Renard, Demoulin, Bourguignon & Hustin, 1979).

Apart from inhibin-like activity, follicular fluids from a variety of species have been shown to contain proteins which inhibit the binding of FSH to the granulosa cell (Darga & Reichert, 1979; Daume, Chari, Hopkinson & Sturm, 1979; Fletcher, Dias, Sanzo & Reichert, 1982; Daume, Chari, Sturm, Hillensjo & Magnusson, 1982; Sluss, Fletcher & Reichert, 1983; Krishnan, Vijayalakshmi & Sheth, 1983; Sluss & Reichert, 1984) and the binding of LH or hCG to thecal cells (Krishnan et al., 1983; Rojas, Cammack, Ruetzel, Ellsworth & Asch, 1986). Follicular fluid proteins have also been reported to prevent the spontaneous maturation of oocytes in vitro (Channing, Anderson & Hoover, 1982a; Schaerf, Anderson & Channing, 1982; Daume et al., 1982), to reduce the steroidogenic activity of cultured ovarian cells (diZerega, Goebelsmann & Nakamura, 1982; diZerega, Marrs, Roche, Campeau & Kling, 1983a; diZerega, Marrs, Campeau & Kling, 1983b; diZerega, Campeau, Nakamura, Ujita, Lobo & Marrs, 1983c; Kling, Roche, Campeau, Nishimura, Nakamura & diZerega, 1984; Kigawa, Ogawa, Matsuoka & Koike, 1986a; Kigawa, Ogawa, Miyamura & Iino, 1986b), to decrease the follicular mitotic index (Cahill, Driancourt, Chamley & Findlay, 1985), and to diminish the RNA synthesis and LH receptor induction (Bernard & Psychoyos, 1977; Osteen, Anderson, Reichert & Channing, 1985) required for luteinisation.

Other properties of ovarian proteins include the stimulation of

pituitary gonadotrophin (Ying, Ling, Bohlen & Guillemin, 1981) and ovarian progesterone secretion (Ledwitz-Rigby, Petito, Tyner & Rigby, 1985), the promotion of follicular angiogenesis (Culler, Tarlatzis, Lightman, Fernandez, Decherny, Negro-Vilar & Naftolin, 1986), and the induction of the acrosome reaction in human spermatozoa (Suarez, Wolf & Meizel, 1986). Recently, a liver-labile ovarian factor, which did not display inhibin-like activity, has been reported to decrease the sensitivity of the rat pituitary gland to GnRH (de Koning, Tijssen & van Rees, 1987). Most of the above proteins are uncharacterised, and considerable overlap may exist in the biological activity of any one preparation. The activity that is of particular interest to this thesis is that of inhibin (for general reviews, see: Chari, 1977; Franchimont, Demoulin, Verstraelen-Proyard, Hazee-Hagelstein, Walton & Waites, 1978; de Jong, 1979; Blanc, 1980; Anderson & DePaolo, 1981).

1.1.4.1 The Assay of Inhibin

One prerequisite for the purification and elucidation of the mechanism of action of a novel hormone is the availability of a reliable assay system. The lack of such an assay is, perhaps, one reason why it has taken over a decade to isolate and characterise inhibin using conventional analytical tools.

The first assays were based on the ability of follicular fluid or seminal plasma to decrease circulating concentrations of FSH in the gonadectomised animal (Lee *et al.*, 1974; Hopkinson *et al.*, 1975; Franchimont *et al.*, 1975; de Jong & Sharpe, 1976). The FSH augmentation test for estimating LH biopotency (Steelman & Pohley,

1953) was modified (Chari, Duraiswami & Franchimont, 1976; Ramasharma, Murthy & Moudgal, 1979) by keeping the dose of hCG constant and by varying the pretreatment dose of the inhibin preparation. This model was further adapted by Cummins (1983), who counted the number of ova shed after hCG-induced ovulation in pregnant mice given follicular fluid, and obtained a dose-dependent reduction in ovulation rate. The specificity, sensitivity, precision and practicabilities of these and similar assays have been reviewed by Hudson, Baker, Eddie, Higginson, Burger, de Kretser, Dobos & Lee (1979).

One major disadvantage of these assays is that factors other than inhibin can affect ovarian weight, serum FSH concentrations and ovulation rate. A specific inhibition of FSH release from cultured pituitary cells thus became a preferred assay system, and has been validated by several authors (rat pituitary cells: de Jong, Smith & van der Molen, 1979; Scott, Burger & Quigg, 1980a; Suter, Goldberg, Wheat, Bahr, Dziuk & Schwartz, 1984; Croze & Franchimont, 1984a; mouse pituitary cells: Ramasharma, Sairam & Ranganathan, 1981; sheep pituitary cells: Tsonis, McNeilly & Baird, 1986a). Recent advances in purification procedures (see section 1.1.4.2) have led to the development of radioimmunoassays specific to polypeptide sequences of the presumed inhibin molecule (McLachlan, Robertson, Burger, de Kretser, 1986a; Rivier, Rivier & Vale, 1986).

1.1.4.2 The Purification of Inhibin

The problem outlined above, namely, the lack of specificity of the earlier inhibin assays, is one reason why attempts to purify inhibin were so often without complete success. Another is the apparent anomolous behaviour of inhibin in conventional protein separation techniques, and a third is the possible existence of sex and species differences in the structure of the molecule.

A large number of purification procedures have been applied to a large number of inhibin sources, and have been extensively reviewed elsewhere (de Jong, Jansen & van der Molen, 1981; de Jong & Robertson, 1985). Gel filtration of testicular or seminal plasma preparations have resulted in molecular weight estimations of greater than 90kDa (Baker, Eddie, Hudson & Niall, 1985; Li, Hammonds, Ramasharma & Chung, 1985), several estimates of 10-20kDa (Chari, Duraiswami & Franchimont, 1978; Thakur, Vaze, Dattatreyamurty, Arbatti & Sheth, 1978; Sheth, Arbatti, Carlquist & Jornvall, 1984a) and the more commonly cited estimate of less than 5kDa (Moodbidri, Joshi & Sheth, 1976; Bandivdekar, Vijayalakshmi, Moodbidri & Sheth, 1982). Ramasharma, Sairam, Seidah, Chretien, Manjunath, Schiller, Yamashiro & Li (1984) incorporated high-pressure liquid chromatography (HPLC) into their programme and proposed a 31-amino acid sequence from a molecule with a molecular weight of less than 5kDa. This peptide was synthesised (Yamashiro, Li, Ramasharma & Sairam, 1984) and was found to be active in a mouse pituitary cell bioassay. Further to this, two molecules were isolated from human seminal plasma with molecular weights of 52 and 92kDa (Li *et al.*, 1985), both of which contained the 31-amino acid sequence found by Ramasharma *et al.* (1984).

In contrast to the low molecular weight material of male origin, early attempts to purify female inhibin resulted in activity with molecular weight estimates from 25kDa (Chari, Hopkinson, Daume & Sturm, 1979) to 140kDa (Godbout & Labrie, 1984), with few estimates less than 5kDa (Vijayalakshmi, Bandivdekar, Joshi, Moodbidri & Sheth, 1980). An efficient affinity matrix was reported (Jansen, Steenbergen, de Jong & van der Molen, 1981; de Jong, Steenbergen, van Dijk & van der Molen, 1982), which gave a bovine preparation of 60-70kDa. The addition of this affinity matrix to HPLC and fast protein liquid chromatography (FPLC) steps suggested similar molecular weight proteins in bovine (de Jong, van Dijk, Steenbergen & van der Molen, 1984; van Dijk, de Jong & van der Molen, 1984; van Dijk, Steenbergen, de Jong & van der Molen, 1985) and porcine follicular fluids (Sairam, Kato, Manjunath & Ramasharma, 1984).

The introduction of HPLC and FPLC steps also indicated the presence of inhibin activity in proteins with molecular weights of 31kDa (Miyamoto, Hasegawa, Fukuda, Nomura, Igarashi, Kangawa & Matsuo, 1985) and 10-12kDa (Rivier, McClintock, Spiess, Vaughan, Dalton, Corrigan, Azad & Vale, 1984). Further, it was reported that a 58kDa bovine follicular fluid preparation could be reduced, by treatment with mercaptoethanol, to two active proteins of 44 and 14kDa (Robertson, Foulds, Leversha, Morgan, Hearn, Burger, Wettenhall & de Kretser, 1985). Modifications to this procedure led to the isolation of a 31kDa protein from the 58kDa starting material, which in turn reduced to subunits of 20 and 14kDa (Forage, Ring, Brown, McInerney, Cobon, Gregson, Robertson, Morgan, Hearn, Findlay, Wettenhall, Burger & de Kretser, 1986; Robertson, de Vos, Foulds, McLachlan, Burger, Morgan, Hearn & de Kretser, 1986a). These 20 and 14kDa proteins were termed the α- and β-subunits,

repectively.

Other authors reported similar results. Fukuda, Miyamoto, Hasegawa, Nomura, Igarashi, Kangawa & Matsuo (1986) isolated active proteins of 96, 55 and 32kDa from bovine follicular fluid, and their 32kDa protein was further separated into two subunits of approximately 20 and 13kDa. As an extension of this work, an antibody was raised against the 32kDa protein; this antibody strongly cross-reacted with the 20 and 13kDa subunits, as well as proteins of approximately 120, 108, 88, 65 and 55kDa molecular weights (Miyamoto, Hasegawa, Fukuda & Igarashi, 1986). Thus the basic inhibin molecule was suggested to be two subunits which aggregated, or bound to larger carrier proteins, *in vivo*.

When similar treatments were applied to porcine follicular fluid, two distinct inhibin-like proteins were obtained, both with molecular weights of 32kDa, and were named inhibins A and B (Ling, Ying, Ueno, Esch, Denoroy & Guillemin, 1985). Each of these two inhibins was reduced to subunits with approximate molecular weights of 18 and 14kDa (Ling *et al.*, 1985; Mason, Hayflick, Ling, Esch, Ueno, Ying, Guillemin, Niall & Seeburg, 1985), similar to the bovine α- and β- subunits of Forage *et al.* (1986). Amino acid sequencing of these subunits suggested that the α-subunits of the A and B inhibins were identical (Mason *et al.*, 1985).

The subunits reported above were used to construct DNA probes, which identified the precursor molecules of the porcine α- and β-subunits as 39kDa and 48kDa proteins, respectively (Mason *et al.*, 1985; Mayo, Cerelli, Spiess, Rivier, Rosenfeld, Evans & Vale, 1986). These DNA probes were also used to isolate human inhibin, which was found to occur as A and B forms, each with α- and β-subunits (Mason, Niall & Seeburg, 1986; Mayo *et al.*, 1986).

Further, the α-subunit was common to both the A and B inhibins, and was homologous to the porcine α-subunit. The A inhibin β-subunit was different to that of the B inhibin, and both β-subunits were very similar to their porcine equivalents (Mason et al., 1986). It has since been shown that the two human subunits arise from separate genes (Stewart, Milborrow, Ring, Crowther & Forage, 1986).

Whilst separate A and B inhibins have yet to be reported for the cow, Stewart et al. (1986) found the bovine α-subunit to be virtually homologous with those of the A and B inhibins of the human and pig, and found homology between the bovine β-subunit and the human A inhibin β-subunit, but not with the human B inhibin β-subunit. This suggests that the cow does not secrete a B inhibin, but these results have yet to be clarified. The inhibin β-subunits have also been shown to be similar in structure to transforming growth factor-β (TGF-β; Derynck, Jarrett, Chen, Eaton, Bell, Assoian, Roberts, Sporn & Goeddel, 1985; Mason et al., 1985); TGF-β was shown to be a potent stimulator of FSH secretion from pituitary cell cultures (Ying, Becker, Baird, Ling, Ueno, Esch & Guillemin, 1986a), and a stimulator of FSH-induced formation of LH receptors on the granulosa cell (Dodson & Schomberg, 1987).

Two laboratories simultaneously published evidence that the β-subunits could form both a heterodimer (Ling, Ying, Ueno, Shimasaki, Esch, Hotta & Guillemin, 1986a) and an A inhibin homodimer (Vale, Rivier, Vaughan, McClintock, Corrigan, Woo, Karr & Spiess, 1986) which, like TGF-β, could stimulate the release of FSH from pituitary cell cultures. These dimers were named activin and FSH-releasing protein, respectively. The A inhibin homodimer of Vale et al. (1986) has also been reported by Ling, Ying, Ueno, Shimasaki, Esch, Hotta & Guillemin (1986b), who termed this form

'homo-activin-A' to distinguish it from 'hetero-activin'. The existence of a 'homo-activin-B' has not yet been reported.

Confirmation and extensions of these studies are awaited, as is the wider availability of a pure preparation; all studies on the actions and secretion of inhibin to date have been conducted with crude materials, and several discrepancies exist, as reviewed below.

1.1.4.3 Actions of Inhibin

As already indicated, the major biological activity of an inhibin preparation is the suppression of FSH secretion. This activity has been demonstrated in the follicular fluid of all mammalian species so far examined (see review by Channing, Gordon, Liu & Ward, 1985a), and short term administration of follicular fluid sources of inhibin have been shown selectively to suppress circulating FSH concentrations in intact (rat: Schwartz & Channing, 1977; Welschen, Hermans & de Jong, 1980; DePaolo, Anderson & Hirshfield, 1981; Kimura, Katoh, Taya & Sasamoto, 1983; ewe: McNeilly, 1984; Henderson, Prisk, Hudson, Ball, McNatty, Lun, Heath, Kieboom, McDiarmid, 1986; Wallace & McNeilly, 1986; monkeys: Channing, Anderson & Hodgen, 1979; Hodgen, Channing, Anderson, Gagliano, Turner & Stouffer, 1980; Channing, Anderson, Hoover, Gagliano & Hodgen, 1981a; Stillman, Williams, Lynch & Hodgen, 1983; cow: Braun, Schams, Buck & Leidl, 1983; Quirk & Fortune, 1985, 1986; pig: Guthrie, Bolt, Kiracofe & Miller, 1985; mare: Bergfelt & Ginther, 1985) and ovariectomised animals (rat: Marder, Channing & Schwartz, 1977; Welschen, Hermans, Dullaart & de Jong, 1977; Koiter, van der Schaaf-Verdonk, Kuiper, Pols-Valkhof &

Schuiling, 1983; Charlesworth, Grady, Shin, Vale, Rivier, Rivier & Schwartz, 1984; Thomas & Nikitovitch-Winer, 1984; ewe: Medhamurthy, Carruthers & Manns, 1985; Findlay, Gill & Doughton, 1985; Campbell, Scaramuzzi, Cognie & Downing, 1986; Clarke, Findlay, Cummins & Ewens, 1986b; heifer: Ireland, Curato & Wilson, 1983; Kiracofe, Ramirez-Godinez, McGowan & Bolt, 1983; rabbit: Patritti Laborde, Rettori & Asch, 1984; Mills, Stopper, Setty & Sorrell, 1986; horse: Miller, Wesson & Ginther, 1979a, 1981), and to inhibit basal FSH secretion from pituitary cells in culture (rat: de Jong, Welschen, Hermans, Smith & van der Molen, 1978; Scott et al., 1980a; Massicotte, Lagace, Godbout & Labrie, 1984; Shiraishi, 1986; Robertson, Giacometti & de Kretser, 1986b; sheep: Huang & Miller, 1984; Tsonis et al., 1986a; Tsonis, Hassan, Martin & McNeilly, 1986b).

Further, antibodies raised against impure (Channing, Tanabe, Turner & Hodgen, 1982b; Al-Obaidi, Bindon, Hillard, O'Shea & Piper, 1984; Lee, Kraft, Atkins & Burger, 1986a; van Dijk, Steenbergen, Gielen & de Jong, 1986) and polypeptide (Rivier et al., 1986) preparations have been shown to neutralise the inhibin activity of exogenous follicular fluid in vivo, and to increase pituitary FSH secretion in vitro, with no reported effects on LH release.

Inhibin preparations have been reported selectively to suppress or abolish the preovulatory and secondary FSH surges in intact rats (Hoak & Schwartz, 1980; Rush, Ashira, Lipner, Williams, McRae & Blake, 1981; Sasamoto, Otani & Shirota, 1981), and the GnRH-induced FSH surge in ovariectomised rats (Koiter et al., 1983; Charlesworth et al., 1984) and pituitary cell cultures (Rush & Vernon, 1985; Robertson et al., 1986b). Some authors report no effect of bovine follicular fluid on GnRH-induced FSH release in the ovariectomised

ewe (Medhamurthy et al., 1985) or intact cow (Braun et al., 1983).

There is evidence indicating that follicular fluid preparations can decrease GnRH-stimulated LH, as well as FSH release in pituitary cell cultures (rat: Croze & Franchimont, 1984a; Massicotte et al., 1984; Shiraishi, 1986; mouse: Kato, Sairam & Ramasharma, 1983), ovariectomised sheep (Knight, Gilbert, Linsey, Castillo & Hostford, 1986; Martin, Wallace, Taylor, Fraser, Tsonis & McNeilly, 1986) and intact rabbits (Mills et al., 1986), and block the preovulatory LH surge in rats if administered at a critical time during pro-oestrus (Rush et al., 1981). Porcine follicular fluid was shown to reduce LH pulse amplitude in the ovariectomised monkey (Schenken, Asch & Anderson, 1984) and in the oestradiol-implanted ovariectomised rat (Babu, Bona-Gallo & Gallo, 1986).

The ability of follicular fluid to suppress GnRH-stimulated LH release was further studied in the ovariectomised ewe with complete hypothalamus-pituitary disconnection, and administered GnRH pulses every two hours (Clarke, Findlay & Cummins, 1984b; Clarke et al., 1986b). Treatment with ovine follicular fluid not only inhibited FSH secretion in these ewes, but also significantly reduced LH pulse amplitude. In contrast, Huang & Miller (1984) reported that ovine follicular fluid enhanced, rather than inhibited, GnRH-induced LH secretion in sheep pituitary cell cultures.

Taken together, the above data suggest that the inhibin-like activity found in follicular fluid suppresses GnRH-independent FSH secretion, and, possibly only in higher doses, reduces the sensitivity of the pituitary gland to GnRH. However, the LH-suppressing activity may be caused by the contamination of the test preparation with other bioactive proteins, as suggested by de Greef, de Jong, de Koning, Steenbergen & van der Vaart (1983).

The possibility of a hypothalamic site of action of inhibin has been investigated, but the evidence is scarce and contradictory (pro: Condon, 1981; Lumpkin, Negro-Vilar, Franchimont & McCann, 1981; Condon, Leipheimer & Curry, 1983; contra: Rush, 1986; Clarke et al., 1986b). It has also been suggested that inhibin may act by specifically increasing the metabolic clearance rate of FSH (van der Schaaf-Verdonk, Harryvan, Schuiling & Koiter, 1984), but this has not been confirmed by other reports (de Greef et al., 1983; McNeilly, 1985).

In addition to the suppressive action of inhibin upon GnRH-induced LH release, a significant increase in mean circulating LH concentrations was noted in porcine follicular fluid treated, ovariectomised mice (Bronson & Channing, 1978). Prolonged administration of bovine follicular fluid also caused an increase in mean LH concentrations in intact rats (de Jong et al., 1978) and sheep (Henderson et al., 1986), and an increase in LH pulse frequency and amplitude in sheep (Wallace & McNeilly, 1985, 1986; Martin et al., 1986). This is unlikely to be a direct effect of inhibin on the pituitary gland, but more likely a consequence of reduced follicular growth and development. This is reflected by the delay in the onset of oestrus and ovulation observed in follicular fluid treated sheep (Miller, Critser, Rowe & Ginther, 1979b; Cummins, 1983; McNeilly, 1984, 1985; Wallace & McNeilly, 1985, 1986; Henderson et al., 1986), monkeys (Stillman et al., 1983), horses (Bergfelt & Ginther, 1985) and heifers (Miller et al., 1979b; Johnson & Smith, 1985; Quirk & Fortune, 1985, 1986). The reduction in circulating FSH concentrations induced by follicular fluid treatment has also been associated with a reduction in the number of large ovarian follicles (rat: Hoak & Schwartz,1980; sheep: Miller

et al., 1979b; Wallace, McNeilly & Tsonis, 1985a; pig: Guthrie et al., 1985), abnormal granulosa cell development (sheep: Wallace, McNeilly & Tsonis, 1985b; Henderson et al., 1986; monkey: Channing et al., 1979) and the inhibition of the ovarian weight increase following unilateral ovariectomy (sheep: Campbell et al., 1986; prepuberal heifers: Johnson, Smith & Elmore, 1985). This decrease in ovarian activity might result in a decrease in oestradiol secretion, which could, in turn, increase circulating LH concentrations, as reported above.

A decrease in oestradiol secretion could also be caused by a direct effect of inhibin on the ovary. Partially purified porcine follicular fluid was shown to inhibit FSH-stimulated oestradiol secretion by cultured granulosa cells from immature, hypophysectomised, diethylstilboestrol treated rats (Kling et al., 1984; Ying, Becker, Ling, Ueno & Guillemin, 1986b). Inhibin preparations were demonstrated to decrease human menopausal gonadotrophin (hMG)-stimulated oestradiol secretion in rats (Chari, Aumuller, Daume, Sturm & Hopkinson, 1981) and in cultured porcine granulosa cells (Chari, Daume, Sturm, Vaupel & Schuler, 1985), and to decrease the number of large antral follicles in PMSG-treated ewes (Cahill, Clarke, Cummins & Findlay, 1984). Recently, TGF-β (which has the opposite effects on FSH secretion to inhibin) has been shown to stimulate the FSH-induced formation of LH receptors on rat granulosa cells (Dodson & Schomberg, 1987). Whilst these preparations were not designed to contain the low molecular weight FSH-binding inhibitor (see above), at least some contamination is possible.

There is a further consequence of the administration of inhibin-enriched materials in vivo. On the cessation of treatment,

FSH has been shown to 'rebound' to concentrations higher than those of untreated control sheep (Miller, Critser & Ginther, 1982; McNeilly, 1984, 1985; Wallace & McNeilly, 1985; Wallace, McNeilly & Baird, 1985c; Henderson et al., 1986), heifers (Quirk & Fortune, 1985, 1986) and mares (Bergfelt & Ginther, 1985), and, when timed to coincide with the early follicular phase of the oestrous cycle, resulted in an increased ovulation rate in sheep (Wallace & McNeilly, 1985; Wallace et al., 1985c; Henderson et al., 1986).

As described in section 1.1.3.3, oestradiol and progesterone are believed to exert a synergistic effect on the secretion of the gonadotrophins. It is of interest to ascertain if inhibin plays an interactive role with these steroids. Bronson & Channing (1978) demonstrated that the administration of either oestradiol or porcine follicular fluid decreased FSH concentrations in ovariectomised mice, and that a combination of the two was no more effective than were either hormone alone. However, in ovariectomised rats, the administration of oestradiol and porcine follicular fluid decreased serum FSH concentrations to less than those of rats given either oestradiol or inhibin (Campbell & Schwartz, 1979). This result could not be confirmed by Rush et al. (1981), but similar results have been obtained in ovariectomised mares given oestradiol in combination with equine follicular fluid (Miller et al., 1981).

It has also been reported that the addition of oestradiol to rat pituitary cells in culture increased both GnRH-induced LH and FSH secretion, and basal FSH secretion, an effect which could be completely inhibited by porcine follicular fluid (Massicotte et al., 1981). The physiological importance of this effect is obscure.

In an attempt to evaluate interactions between inhibin and oestradiol in sheep, ovariectomised ewes were passively immunised

against GnRH, and treated with bovine follicular fluid in the presence or absence of an oestradiol implant (Martin et al., 1986). Unfortunately, the effects of inhibin and oestradiol on FSH secretion could not be tested as the follicular fluid alone reduced serum FSH concentrations to the limit of detection in this animal model.

Whilst much remains to be done on this aspect, it is apparent that inhibin is important in the differential regulation of LH and FSH secretion. It is thus of importance to consider also those factors which control the secretion of inhibin.

1.1.4.4 The Secretion of Inhibin

That the gonad is the major source of inhibin in mammals is deduced from the presence of biological activity in follicular and testicular fluids (vide supra). In the female, fluctuations in inhibin-like activity in follicular fluid are reported at different stages of the oestrous cycle, with higher levels at pro-oestrus in rats (Fujii, Hoover & Channing, 1983), hamsters (Chappel, 1979) and humans (Channing, Tanabe, Guthrie, Batta & Hoover, 1981b; Sheth, Vaze, Thakur, Arbatti, Hazari, Mehta & Joshi, 1981), but the reverse has been reported for heifers (Ireland et al., 1983) and pigs (Suter, Bahr, Dziuk & Schwartz, 1986).

The large antral follicle secretes the greater part of the inhibin content of the ovary (rat: Taya, Kimura & Sasamoto, 1984; Sander, Meijs-Roelofs, van Leeuwen, Kramer & van Cappellen, 1986; sheep: Scott, Quigg, Trounson, Tsonis & Findlay, 1980b; Findlay, Tsonis, Staples & Cahill, 1986; cow: Henderson, Franchimont,

Charlet-Renard & McNatty, 1984b; Padmanabhan, Convey, Roche & Ireland, 1984; human: Channing, Gagliano, Tanabe, Fortuny & Cortes-Prieto, 1985b), with very little being secreted by atretic (cow: Henderson et al., 1984b; Franchimont & Valcke, 1986) or cystic follicles (human: Daume, Chari, Hopkinson, Sturm & Hirschhauser, 1978; Channing et al., 1981b). In the human, significant positive correlations were reported between ovarian inhibin and oestradiol secretion, and between inhibin secretion and the number of follicles present in the ovary (McLachlan, Robertson, Healy, de Kretser & Burger, 1986b). Within the non-atretic follicle, the granulosa cells are the major contributors of inhibin (rat: Erickson & Hsueh, 1978b; Sander, van Leeuwen & de Jong, 1984; Lee, 1984; cow: Henderson & Franchimont, 1981, 1983; Rokukawa, Inoue, Miyamoto, Kurosumi & Igarashi, 1986; pig: Massicotte et al., 1984; Rokukawa et al., 1986; monkey: Channing et al., 1982a).

Inhibin-like activities have also been reported in human placental tissue (Bandivdekar, Vijayalakshmi, Jaswaney & Sheth, 1981; Bandivdekar, Varadkar & Sheth, 1985; McLachlan, Healy, Robertson, Burger & de Kretser, 1986c), gastric juice (Sheth, Vaze & Sheth, 1982) and in the serum of cancer patients (Sheth, Hurkadli, Sathe & Sheth, 1984b). The physiological significance of these reports is unknown. Recently, Tsonis, Hillier & Baird (1987) have shown that human granulosa-lutein cells secrete an inhibin-like activity.

The secretion of inhibin, like that of oestradiol, is stimulated by FSH or PMSG (mouse: Lee & Gibson, 1985; rat: Lee & Findlay, 1982; Lee, McMaster, Quigg & Leversha, 1982; Lee, Colvin, McMaster & Quigg, 1984; Lee, Zhang, Carson & Burger, 1986b; Bicsak, Tucker,

Cappel, Vaughan, Rivier, Vale & Hsueh, 1986; Tsukamoto, Taya, Watanabe & Sasamoto, 1986; Rivier et al., 1986; Davis, Dench, Nikolaidis, Clements, Forage, Krozowski & Burger, 1986; cow: Henderson et al., 1984b; Franchimont & Valcke, 1986; sheep: Findlay et al., 1986), but not by LH or hCG (Lee et al., 1982, 1986b) unless the granulosa cells have been pretreated with FSH to induce the formation of LH receptors (Bicsak et al., 1986).

There is evidence that the steroids may participate in the regulation of inhibin secretion (Croze & Franchimont, 1984b). Oestradiol and androstenedione have been reported to enhance, and progesterone to inhibit PMSG-stimulated inhibin secretion from cultured rat granulosa cells (Lee et al., 1984, 1986b). In the cultured bovine granulosa cell, aromatisable and non-aromatisable androgens (testosterone and 5α-dihydrotestosterone, respectively) were both shown to stimulate inhibin secretion (Franchimont, Croze, Henderson, Hazee-Hagelstein & Lecomte-Yerna, 1984), whilst progesterone reduced inhibin secretion and oestradiol was without effect (Henderson & Franchimont, 1981). It is of interest to note that FSH stimulated inhibin release from healthy, but not atretic follicles, whereas testosterone stimulated inhibin secretion from atretic as well as healthy follicles (Henderson et al., 1984b), which could implicate inhibin in the process of follicular atresia. This remains to be properly tested.

1.2 CONTROL OF OVULATION RATE

The previous sections have described the major endocrine factors which govern ovarian activity, and how these, in turn, are controlled. This section reviews the ways in which this information has been used to manipulate ovarian activity, and the consequences thereof.

1.2.1 OCCURRENCE & IMPLICATIONS OF MULTIPLE BIRTHS IN CATTLE

The frequency of twinning in cattle, and the subsequent effects on the productivity of both calves and dams, has been well studied (Hendy & Bowman, 1970; Morris, 1984a).

Rutledge (1975) reviewed the literature on the frequency with which twins occur in cattle, and gave estimates which vary from 0.0% to 11.3% (both estimates were for the same breed, the Brown-Swiss). The range for Friesian cattle was 0.5 to 3.0%. The twinning rates for beef breeds were, in general, lower than those of dairy breeds (Johansson, 1932; Rutledge, 1975). Few data are available on the incidence of triplet births; two estimates in the literature gave 0.03% in a Holstein-Friesian herd (Erb & Morrison, 1959) and 0.005% in an Indian herd (Bhattacharya, Prabhu & Chatterjee, 1956). Little is known about the occurrence of monozygotic twins; Johansson & Venge (1951) estimated that 10% of like-sexed twins were monozygotic, although earlier reports suggested a lower figure (Gowen, 1922; Johansson, 1932).

As a genetic trait, twinning in cattle was reported to have a low heritability (0.006 to 0.05: Bar-Anan & Bowman, 1974; Cady &

Van Vleck, 1978; Horn, 1983; Syrstad, 1984) and low repeatability (0.04 to 0.07: Korkman, 1948; Horn, 1983) compared with those of sheep (approximately 0.16 for both: Fogarty, Dickerson & Young, 1982). Twinning rates and the heritability of twinning were reported to increase with the age and parity of the dam (Pfau, Bartlett, Shuart, 1948; Rutledge, 1975; Cady & Van Vleck, 1978; Syrstad, 1984).

The implications of twinning, for both dam and progeny, are of practical importance. Cows bearing twins were reported to have a shorter period of gestation (by 5 to 7 days: Pfau et al., 1948; Tantawy & Ahmed, 1957; Cady & Van Vleck, 1978), and an increased incidence of abortion, dystocia and subsequent reproductive disorders (Clapp, 1934; Tantawy & Ahmed, 1957; Erb & Morrison, 1959; Boyd & Reed, 1961; Comberg & Velten, 1962; Brodauf, 1963; Labhsetwar, Tyler & Casida, 1963; Callahan, Erb, Surve & Randel, 1971; Cady & Van Vleck, 1978; Langley, 1983). The mortality of twin calves (from birth to 2 days of age) was higher than that of singletons (Johansson, 1932; Pfau et al., 1948; Comberg & Velten, 1962; Brodauf, 1963). However, some authors have reported no increase in calving difficulties or subsequent reproductive disorders (Labhsetwar et al., 1963; Turman, Laster, Renbarger & Stephens, 1971; Bellows, Short, Urick & Pahnish, 1974), and the mortality of the calves to be higher only in cases of triplet or higher multiple births (Hewitt, 1934; Turman et al., 1971; Owens, Edey, Bindon & Piper, 1985).

Twin calves were shown to be significantly smaller at birth than were singletons (Johansson, 1932; Tantawy & Ahmed, 1957; Kay, Little & Kitchenham, 1976; Cady & Van Vleck, 1978; Smith, Pollack & Anderson, 1982), although this difference had decreased by the age

of puberty in most reports (Tantawy & Ahmed, 1957; Bellows et al., 1974; Kay et al., 1976). Where twin calves were found to be lighter than singletons at weaning, the combined weight of the twins was significantly higher than that of the single-born calf (Smith et al., 1982). It has been shown that dams with twin calves suckled less than dams with single calves, as the calves grew older, thus forcing twin calves to forage earlier than singletons (Price, Martinez & Coe, 1985).

The remaining major implication of twinning in the cow is the production of the freemartin. It has been known for centuries "that when a cow brings forth two calves, and that one of them is a bull-calf, and the other a cow to appearance, the cow-calf is unfit for propagation; but the bull-calf becomes a very proper bull. They are known not to breed: they do not even fhew the leaft inclination for the bull, nor does the bull ever take the leaft notice of them [sic.]" (Hunter, 1779).

Freemartins were first thought to arise from monozygotic twins (Cole, 1916), but Lillie (1922) described the formation of placental anastomoses between dizygotic twins. These anastomoses were reported to form in early pregnancy (Hafez & Rajakoski, 1964), and are the route through which humoral or cellular influences pass from the male foetus to the female, to inhibit the differentiation of the genital tract (see Marcum, 1974; Kastli & Hall, 1978).

Not all females born twin-to-bull are freemartins. Johansson (1932) and Hewitt (1934) each reported that approximately 7.5% of such females were fertile. Since the average sex ratio was reported to be 1:2:1 for male-male, male-female, and female-female twins, respectively (Johansson, 1932), about one half of all twin calves would be female, and one half of these would be born twinned with a

bull. Thus, approximately 47% of all female twins would be sterile. This presents an economic loss to a dairy herd, but a gain for a beef herd; these economics have been discussed by Bowman (1976).

1.2.2 THE INDUCTION OF MULTIPLE BIRTHS

1.2.2.1 Genetic Selection

Based on the advantages and disadvantages of twins described above, several authors discouraged (Pfau et al., 1948; Erb & Morrison, 1959; Brodauf, 1963; Cady & Van Vleck, 1978) whilst others encouraged (Hendy & Bowman, 1970; Rutledge, 1975; Kay et al., 1976) selection in favour of twinning.

Selection for multiple births was practised in sheep with considerable success (Quirke, Bradford, Famula & Torrell, 1985), and led to, amongst others, the Booroola strain of Merino (Turner, 1978), which is believed to be influenced by a major gene acting on both ovulation rate and litter size (Piper & Bindon, 1982). An important distinction should be made here; the most practical selection criterion is litter size, although Piper, Bindon, Atkins & Rogan (1984) argued for selection based on ovulation rate, the heritability of which was reported to be higher than that of litter size. Litter size is not only affected by ovulation rate, but also by other physiological, nutritional and genetical factors (Wilmut, Sales & Ashworth, 1985). The genetics of litter size in sheep has been reviewed (Bradford, 1985). In one study, the incidence of

cattle with two corpora lutea was 13%, but the incidence of twin births in the same animals was significantly lower at 2% (Kidder, Barrett & Casida, 1952).

Selection experiments in cattle have been less successful than those in sheep. After 30 years of selection in an Aberdeen Angus herd, the mean twinning rate was no different from that of an unselected neighbouring herd (Mechling & Carter, 1964). A herd whose foundation stock consisted of dams with a history of at least two twin births was established at Reading (Hendy & Bowman, 1970), but was later disbanded without having been successful (J.C.Bowman, personal communication). Similar selection criteria were used to establish a herd in France which started with a twinning rate of 23%, but which had fallen to 11% in a subsequent generation (Frebling, Gillard & Menissier, 1982), and another herd in the United States, whose mean twinning rate fell from 16.4% in the foundation dams to 7.8% in later progeny (Gregory, Echternkamp, Cundiff, Koch & Dickerson, 1986). A herd of twinning cattle has been collected in New Zealand (Morris, 1984b), although the current twinning rate in this herd (7%) is caused mainly by the retention of collected dams, rather than the performance of daughters (C.A.Morris, personal communication).

It was suggested that it should be possible to generate, in 10 years, a herd of cattle with a twinning rate of 20% (Maijala & Syvajarvi. 1977); a suggested reason for the failure to achieve this to date, is that the initial selection process was not sufficiently intense (Piper & Bindon, 1979). Prolific bulls have been reported to produce long lines of twin-bearing daughters (Lush, 1925), although these were not used to the best advantage. Whilst no highly prolific breed of cow exists, it has been shown that the

mean twinning rate of a herd of Canadian cattle can be increased by cross-breeding with Brown-Swiss cattle of a higher fecundity (Rony, Fahmy & Holtmann, 1985).

1.2.2.2 Gonadotrophin Treatment

The knowledge that the gonadotrophins exert a marked effect on the ovaries initiated attempts to increase twinning rates in cattle by the administration of pregnant mares' serum (Folley & Malpress, 1945) and PMSG preparations (Hammond & Bhattacharya, 1944; Hammond, 1949). These treatments raised ovulation rates in excess of that required for twinning, and this process became known as superovulation. Several other attempts resulted in the induction of 2 to 4 corpora lutea (Bellows, Anderson & Short, 1969; Turman et al., 1971; Wildt, Woody & Dukelow, 1975), but most were variable and with a high incidence of superovulation (Hafez, Jainudeen & Lindsay, 1965; Laster, Turman, Stephens & Renbarger, 1971; Smith, Sitton & Vincent, 1973).

The superovulatory response has been used specifically to gain a large number of eggs or embryos for transfer into other individuals (Dowling, 1949; Brock & Rowson, 1952; Sreenan & Gosling, 1977; Seidel, Elsden, Nelson & Bowen, 1978; Sreenan, Beehan & Gosling, 1978; Staigmiller, Short, Bellows & Carr, 1979; Lauria, Genazzani, Oliva, Inaudi, Cremonesi, Monittola & Aureli, 1982; McGowan, Johnson, Mapletoft & Jochle, 1983; Voss, Olivera-Angel & Holtz, 1983; Donaldson, Ward & Glenn, 1986; Wubishet, Graves, Spahr & Kesler, 1986). The number of cows producing twins has been increased by the transfer of two embryos to each recipient (see

Gordon, 1976), or by placing an additional embryo into a previously inseminated cow (Diskin & Sreenan, 1984; Kennedy, Boland & Gordon, 1984a, b). The occurrence of freemartins could still be a potential disadvantage of these techniques, unless sexed embryos were transferred as suggested by Picard, King & Betteridge (1985). A further development has been the production of monozygotic twins from bisected embryos (Willasden and Polge, 1981; Willasden, Lehn-Jensen, Fehilly & Newcombe, 1981; Baker, Eberhard, Leffel, Rohde & Henschen, 1984; Williams, Elsden & Seidel, 1984).

The effects of superovulatory treatments on the reproductive system have been investigated. The stimulation of ovarian follicles and corpora lutea by exogenous gonadotrophins increased circulating concentrations of oestradiol and progesterone (Booth, Newcomb, Strange, Rowson & Sacher, 1975; Alcivar, Maurer & Anderson, 1984). Treatment with PMSG or FSH advanced the timing of the preovulatory gonadotrophin surge (Schams, Menzer, Schallenberger, Hoffman, Hahn & Hahn, 1978; Schams, Menzer, Schallenberger, Hoffmann, Prokopp, Hahn & Hahn, 1979; Donaldson, 1985; Yadav, Walton & Leslie, 1986), without effect upon pulsatile LH secretion (Yadav et al., 1986) or the magnitude of the LH surge in some (Schams et al., 1979; Alcivar et al., 1984; Donaldson, 1985), but not all reports (Yadav et al, 1986).

The lack of an effect of superovulatory treatments on LH concentrations could be of importance. The number of fertilised ova recovered was reduced if superovulation was induced with preparations containing LH, in comparison to treatments without LH, in heifers (Chupin, Combarnous & Procureur, 1984; Donaldson et al., 1986) and rats (Opavsky & Armstrong, 1985); Donaldson (1985) suggested that FSH is responsible for the quantity of eggs produced,

and LH for the quality of the eggs. It is interesting to note that passive immunisation of ewes against (bovine)LH increased circulating FSH concentrations and ovulation rate (Fitzgerald, Ruggles & Hansel, 1985).

1.2.2.3 Manipulation of Ovarian Feedback

Another approach is to increase gonadotrophin secretion by decreasing ovarian hormone secretion. The use of a non-steroidal anti-oestrogen increased ovulation rate in sheep (Land & Scaramuzzi, 1979), but the effects were small & inconsistent.

Active immunisation against oestradiol blocked ovulation in rats (Hillier, Groom, Boyns & Cameron, 1975; Kaushansky, Bauminger, Koch & Lindner, 1977), monkeys (Schwartz, Dyrenfurth, Khalaf, Vande Wiele & Ferin, 1975), sheep (Scaramuzzi, Baird, Clarke, Davidson, Martensz & Van Look, 1977; Rawlings, Kennedy & Henricks, 1978; Scaramuzzi, Martensz & Van Look, 1980a) and cattle (Martin, Henricks, Hill & Rawlings, 1978). These effects were associated with a high incidence of ovarian cysts (e.g., Hillier et al., 1975; Martin et al., 1978; Scaramuzzi et al., 1980a), and increased circulating concentrations of LH (Hillier et al., 1975; Pant, Dobson & Ward, 1978) and FSH in some cases (Pant et al., 1978).

Active immunisation of sheep against oestrone has been reported both to block ovulation (Scaramuzzi et al., 1980a), and to increase ovulation (Scaramuzzi et al., 1977; Scaramuzzi, Cox & Hoskinson, 1982; McMillan, Smith & Kitney, 1984) and lambing rates (Smith, Cox, McGowan, Wilson & Hoskinson, 1981; Cox, Wilson, Scaramuzzi, Hoskinson, George & Bindon, 1982). This treatment has been reported

to have no effect on ovulation rate in heifers (Sreenan, Diskin, Morris, Tait & Kilpatrick, 1983). An increase in ovulation rate has been reported in ewes actively immunised against progesterone (Thomas et al., 1984a), although these ewes also displayed a significantly reduced incidence of behavioural oestrus.

Similar experiments have been conducted with the androgens. Active immunisation against testosterone was shown to block ovulation in rats (Hillier et al., 1975) and in some reports in sheep (Scaramuzzi et al., 1977; Scaramuzzi, 1979; Scaramuzzi, Baird, Martensz, Turnbull & Van Look, 1981), whilst increases in ovulation rate were also described in sheep (Cox et al., 1982). In the experiments of Hillier et al. (1975), the immunised rat showed a high incidence of ovarian cysts and elevated FSH concentrations. In cattle, immunisation against testosterone enhanced the superovulatory effect of PMSG, but lowered the rate of egg recovery (Boland, Nancarrow, Hoskinson, Murray, Scaramuzzi, Radford, Avenell & Bindon, 1985). A preliminary report has appeared recently, which described double ovulations in 2 of 7 heifers immunised against testosterone, and a low incidence of anoestrus (D'Occhio, Gifford, Cox, Weatherly & Setchell, 1986).

Immunisation against androstenedione consistently increased ovulation rate (Scaramuzzi et al., 1977, 1982; Van Look, Clarke, Davidson & Scaramuzzi, 1978; Scaramuzzi, Baird, Clarke, Martensz & Van Look, 1980b; Quirke, Hanrahan, Kilpatrick & Box, 1983) and litter size in sheep (Cox et al., 1982; Scaramuzzi et al., 1982). This approach has been exploited, and a commercial androstenedione 'vaccine' is now available under the trade name of Fecundin. Large field trials were conducted with Fecundin, and significant increases in twinning rates have been reported (Geldard, Dow & Kieran, 1984;

Harding, Hardy & Joby, 1984). The responses of ewes to Fecundin have been shown to be affected by breed (Quirke, Hanrahan Tait & Kilpatrick, 1986) and nutrition (Cummins, Spiker, Cook & Cox, 1984). The economics of using Fecundin have been discussed (Leyonhjelm, 1984).

A recent study has reported some deleterious effects of immunisation against androstenedione upon fertilisation and embryo survival in sheep (Boland, Nancarrow, Murray, Scaramuzzi, Sutton, Hoskinson & Hazelton, 1986), but not on the incidence of chromosome abnormalities in the embryos (Murray, Boland, Moran, Sutton, Nancarrow, Scaramuzzi & Hoskinson, 1985).

In the cow, increases in ovulation rate have also been obtained with immunisation against androstenedione (Wise & Schanbacher, 1983; Sreenan et al., 1983), although these results cannot be repeated by other authors (Sreenan, Morris, Tait & Diskin, 1987).

One problem with active immunisation is that this method cannot be controlled. With passive immunisation however, the antibody can be characterised (in terms of titre, specificity and avidity) and administered in known quantities (discussed by Webb, Land, Pathiraja & Morris, 1984). Passive immunisation against one or more of the major ovarian steroids increased ovulation rate and litter size in sheep (Pathiraja, 1982; Land, Morris, Baxter, Fordyce & Foster, 1982; Webb et al., 1984; Rhind, Gunn, Morris, Clayton, Leslie & Gittus, 1985; Rhind, Morris, Clayton, Gunn, Gittus & Leslie, 1987), although passive immunisation against oestradiol has been reported to block ovulation in sheep (Fairclough, Smith & Peterson, 1976). Treatment with antisera raised against all four major steroids increased LH pulse frequency in sheep, whilst FSH concentrations were seen to rise only in ewes treated with an anti-oestrogen serum

(Pathiraja, 1982; Pathiraja, Carr, Fordyce, Forster, Land & Morris, 1984).

Preliminary results with passively immunised cattle indicate that the response is more varied than that in sheep, with no overall increase in ovulation rate (Sreenan et al., 1983; Webb et al., 1984).

The above studies have been extended, not by neutralising circulating steroids, but by inhibiting their synthesis. The administration of a 3β-HSD enzyme inhibitor to sheep significantly decreased circulating progesterone concentrations, and increased oestradiol secretion and ovulation rate, without altering the duration of the oestrous cycle (Webb, 1987).

Ovarian protein feedback has also been the subject of manipulation. The administration of inhibin to sheep caused the suppression of circulating FSH concentrations, which rebounded above pretreatment values when the inhibin treatment was stopped. The increase in ovulation rate occurring as a result of this FSH rebound has been decribed (section 1.1.4.3).

Ovulation rate has been increased by actively immunising sheep against follicular fluid inhibin preparations (Cummins, 1983; Henderson, Franchimont, Lecomte-Yerna, Hudson & Ball, 1984c; O'Shea, Al-Obaidi, Hillard, Bindon, Cummins & Findlay, 1984; Cummins, O'Shea, Al-Obaidi, Bindon & Findlay, 1986a). In these studies the number of follicles of diameter >3.5mm in the ovary, and the number of lambs born were also increased. However, no consistent changes in circulating LH or FSH concentrations were reported. Again, only preliminary data are available for the cow, and these have shown that up to half the immunised animals had double ovulations (Cummins, O'Shea & Bindon, 1986b).

The identification of the mechanism(s) which allow the expression of high ovulation rates in prolific breeds could improve techniques aimed at raising litter size. Whilst such studies can be conducted with sheep, observations in cattle have to be limited to individuals with histories of twin births.

Mean LH concentrations were reported to be no different between prolific and less prolific breeds of sheep (Land, Pelletier, Thimonier & Mauleon, 1973; Bindon, Blanc, Pelletier, Terqui & Thimonier, 1979; Webb et al., 1985a; Thomas, Oldham & Martin, 1984b), except for a study which indicated that mean LH concentrations were lower in prolific breeds (Lahlou-Kassi, Schams & Glatzel, 1984). No differences in mean LH concentrations were seen between women with a high frequency of twinning and those without (Nylander, 1973). Prolific breeds of sheep were shown to display a higher LH pulse frequency than were less prolific sheep (Rhind & McNeilly, 1983; Thomas et al., 1984b), although these results are in conflict with those of Martin & Clapin (1982), which showed that increasing the frequency of LH pulses in low fecund ewes did not increase ovulation rate.

The timing of the preovulatory LH surge was reported to be delayed in sheep with higher ovulation rates (Land et al., 1973; Bindon et al., 1979), and the duration of oestrus longer (Land, Thompson & Baird, 1972), in comparison with ewes of lower fecundity.

The results of FSH analyses are mixed. Twinning women were reported to have higher mean FSH concentrations during the follicular phase of the cycle than those of women who bore singletons (Nylander, 1973), whereas no consistent differences were

found in sheep (Bindon et al., 1979; Bindon, Findlay & Piper, 1982; Webb & England, 1982b). There were differences between sheep breeds in pituitary FSH content (Robertson, Ellis, Foulds, Findlay & Bindon, 1984) and the magnitude of the secondary FSH surge (Cahill, Saumande, Ravault, Blanc, Thimonier, Mariana & Mauleon, 1981; Lahlou-Kassi et al., 1984; Bindon, Piper, Cummins, O'Shea, Hillard, Findlay & Robertson, 1985), both these values being higher in prolific ewes. However, when follicular fluid was administered to the highly fecund Booroola strain, and the secondary FSH surge abolished, there was no effect on subsequent ovulation rate (Bindon et al., 1985).

Land (1976) postulated that prolific sheep had similar plasma LH concentrations to low fecund sheep because they were less sensitive to the negative feedback effects of oestradiol, but it has since been shown that breeds that differed in prolificacy, did not have different blood oestradiol concentrations (Baird, Ralph, Seamark, Amato & Bindon, 1982; McNatty, Henderson, Lun, Heath, Ball, Hudson, Fannin, Gibb, Kieboom & Smith, 1985b; Downing, Baird, Campbell & Scaramuzzi, 1986). There is further evidence that differences between breeds are expressed at the ovarian rather than at the pituitary/hypothalamic level. Cattle with a history of twin births were found to be more sensitive to exogenous FSH, with respect to ovulation rate, than were single-bearing cows (Thimonier, Bindon & Piper, 1979; Bindon, Piper, Hillard & Nethery, 1986). In another study, the ovulation rate of hypophysectomised sheep of a prolific breed was higher than that of a less prolific breed, when both breeds were given an identical dose of PMSG (Fry, Clarke, Cummins, Bindon, Piper & Cahill, 1986).

There is reported to be no difference in the total number of

follicles in the ovaries of prepuberal sheep selected for and against multiple births, if the data are corrected for litter size (Tassell, Kennedy, Bindon & Piper, 1983), but the number of antral follicles in highly fecund sheep was found to be higher than that of a less fecund breed by some authors (Land, 1970; Lahlou-Kassi & Mariana, 1984; McNatty, Lun, Heath & O'Keeffe, 1987), whereas other authors describe no significant differences (Driancourt, Cahill & Bindon, 1985; Driancourt, Gauld, Terqui & Webb, 1986).

A correlation between the number of oestrogen active follicles and ovulation rate has been described (Baird et al., 1982; Webb & Gauld, 1985a, b; McNatty, Lun, Heath, Ball, Smith, Hudson, McDiarmid, Gibb & Henderson, 1986a), and a lower rate of follicular atresia was reported in prolific breeds (Driancourt et al., 1985, 1986). The oestrogenic follicles of the prolific Booroola sheep were shown to be smaller and more numerous than those of the less prolific Merino (Baird et al., 1982; McNatty, et al., 1985b, 1986a), and to contain fewer granulosa cells; the total number of granulosa cells per ovary were not different between breeds (McNatty et al., 1986a), thereby there were no breed differences in ovarian oestradiol secretion.

The development of smaller oestrogenic follicles in the Booroola implied that the follicular cells were more sensitive to gonadotrophin stimulation than those of the Merino. There were no breed differences in the ability of all follicular cells to bind hCG or LH, regardless of follicle size (McNatty, O'Keeffe, Henderson, Heath & Lun, 1986b), but small follicles (less than 1mm diameter) from Booroola ewes contained, and secreted in vitro, more cAMP than similar sized follicles from Merino ewes (McNatty, Kieboom, McDiarmid, Heath & Lun, 1986c). The underlying mechanism which

causes this difference is unknown, but it is of interest to note that the ovaries from the Booroola ewes contain significantly less inhibin than those of the Merino (Cummins, O'Shea, Bindon, Lee & Findlay, 1983), despite the similar numbers of granulosa cells.

CHAPTER TWO

MATERIALS AND METHODS

2.1 ANIMALS

The cattle used in these studies were Charolais, Simmental or Hereford-Friesian cross-bred heifers, one to two years of age, as indicated in the relevant experimental chapters. They were all purchased locally and kept under normal husbandry conditions on the farms of the Institute of Animal Physiology and Genetics Research (formerly the Animal Breeding Research Organisation) in Scotland. Routine observations for behavioural oestrus were made at least twice daily, aided by a heat detection device (Kamar Inc., Colorado).

All experimental procedures were performed in compliance with the Cruelty to Animals Act, 1876.

2.2 SURGICAL PROCEDURES

2.2.1 Blood Sampling

Blood samples were taken from the jugular vein by vacutainer or syringe for either plasma or serum collection, as indicated. Plasma was harvested from heparinised samples by centrifugation at 4°C within four hours of collection, whilst serum samples were collected from blood which had been allowed to clot overnight at room temperature prior to centrifugation. All samples were stored at -20°C until required for assay.

To characterise the pulsatile secretion of LH, blood samples

were taken every ten minutes for six, eight or twelve hours via indwelling jugular cannulae. The animals were cannulated one or two days before sampling, as follows. The animals were restrained in a crush without general anaesthesia, & the skin around the vein was shaved and surgically prepared. A small skin incision was made directly over the vein, and a 13 gauge cannula (Intranule; Vygon UK Ltd., Cirencester) was inserted and passed down the vein. Once in place, it was sealed with a sterile membrane cap, flushed with sterile heparinised saline and secured in place with two sutures. The cannulae appeared to cause no discomfort once in place, and could be kept patent for a week with regular flushing.

2.2.2 Laparoscopy

Two methods of laparoscopy were employed in these studies, modified from the techniques of Holland, Bindon, Piper, Thimonier, Cornish and Radford (1981). The mid-ventral approach required the animals to be fasted from food and water for 24 hours. General anaesthesia was induced by intravenous injection of a 10% aqueous solution of sodium thiopentone (1g Intraval Sodium/90kg liveweight; May & Baker, Dagenham) and maintained on 4% halothane (Fluothane; ICI, Macclesfield) in air passed through an endotracheal tube. The animals were suspended in a recumbent position with the hindquarters higher than the forequarters. An area of skin approximately 10-15cm anterior to the mammary gland was surgically prepared, and two small incisions placed either side of the midline, about 20cm apart. The laparoscope trochar and cannula were inserted into the peritoneal cavity through one incision, and the probe trocar and cannula

inserted through the other incision. Medical air was used to inflate the cavity prior to observing the ovaries. After observation, a topical antibiotic was applied to the incision, and the animal was given an intramuscular dose of a long acting penicillin (Duphapen; Duphar Veterinary Ltd., Southampton). Sutures were not normally necessary. The laparoscope used for this technique was 7 or 10mm in diameter and 30cm in length (Richard Wolf (UK) Ltd., Mitcham).

The second, sublumbar, technique required that the animals be fasted for 36 to 48 hours to sufficiently reduce the volume of the rumen. The animals were restrained in a tilted crush, such that the hindquarters were higher than the forequarters; most animals walked up a ramp and stood in the tilted crush without apparent discomfort. Each heifer was lightly sedated (Rompun; Bayer UK Ltd., Bury St. Edmunds), and the sublumbar fossa was surgically prepared. Two discrete sites were infiltrated with 5ml 2% lignocaine (Lignovet; C-Vet Ltd., Bury St. Edmunds: or Lignocaine-A; Univet 2 Ltd., Bicester) to anaesthetise skin, muscle and peritoneum, one site about 10cm ventrally and slightly anterior to the first. Small skin incisions were made, and the trocars and cannulae inserted in a slightly posterior direction. Insufflation was not normally necessary. After observation, similar septic precautions were taken as in the mid-ventral technique, and the animal was led out of the crush. To view both ovaries from one flank, a telescope 60cm in length was required.

To photograph the ovaries, the animals were examined by the mid-ventral approach; a Panoview (Wolf) laparoscope 10mm in diameter and 30cm in length, was used in conjunction with a fluid filled light cable and a 400W halogen metal vapour light source.

2.2.3 Ovariectomy

Ovariectomies were performed in the tilted crush otherwise used for sublumbar laparoscopy. Paravertebral anaesthesia was induced with lignocaine (Hostacain; Hoechst UK Ltd., Milton Keynes), and a flank incision made running ventrally from the sublumbar fossa to a length of approximately 20-30cm. The ovaries were located, the blood supply stopped with double ligatures, and the ovaries removed with either an ecraseur or a scalpel. In most cases, incisions were made on each flank to gain access to each ovary. Degradeable sutures (Dexon; Davis & Geck, Gosport) were used to close the incisions, and the cutaneous sutures were removed after two weeks to prevent infection. After surgery, all animals received a short-acting tetracycline antibiotic injection (Engemycin; Mycofarm Ltd., Braintree) daily for five days. Any cases of infection were treated in accordance with veterinary practice.

2.2.4 Implant Insertion

Oestradiol implants were made from Silastic sheeting (Dow Corning, Reading) of 0.127mm thickness to give sachet sizes of approximately 4 x 6.5cm, 4 x 2.5cm and 3.5 x 1.25cm, excluding the area occupied by the silicone adhesive. Sachets were filled with pure crystalline oestradiol-17β (Sigma Ltd., Dorset) and sealed. The adhesive was allowed 12-24 hours to harden, then the implants were tested for leaks by soaking in methanol for 4 to 6 hours followed by an overnight soak in saline. Before surgery, all implants were incubated in 1% bovine serum albumin in sterile saline at 37°C for 12 hours to remove any remaining surface contamination

and to coat the surface with protein. In a pilot study, this treatment was shown to reduce greatly the 'surge' in plasma oestradiol concentration occurring immediately after implant insertion.

The implants were inserted under the skin overlying the thorax just ventral to the axilla. A subcutaneous pocket was made with a blunt instrument whilst the animals were lightly anaesthetised, and the implant inserted to lie flat. The incision was closed with sutures. The implants were removed at the end of the experiment by a single incision on the opposite side of the implant to the entry incision; all implants were removed intact, and none showed signs of leaking or of having been exhausted of oestradiol.

2.3 HORMONE ASSAYS

The work described in this thesis required analyses of circulating concentrations of LH, FSH, progesterone and oestradiol, as well as culture medium concentrations of oestradiol. The steroid and FSH assays employed were all modified from ovine assays routinely used in the Department, whilst the LH assay was validated using a new antiserum. The validation and routine procedures of these assays follow.

2.3.1 Buffers

Two assay buffers were used in these assays, one for use in the gonadotrophin assays and the other for use in the steroid assays. Both buffers incorporated a 0.5M phosphate base, which was prepared by dissolving 716g disodium hydrogen orthophosphate into 4 litres double distilled deionised water (herein referred to simply as water unless specified otherwise), and 78g sodium dihydrogen orthophosphate into 1 litre water. The sodium dihydrogen salt solution (approximately 640ml) was mixed into the disodium hydrogen salt solution to attain pH 7.5. The buffer was then stored in the dark at room temperature.

A bovine serum albumin general assay diluent (BSA-GAD) was used for the gonadotrophin assays. Two hundred millilitres of the stock phosphate buffer was diluted to 2 litres in water into which had been dissolved 18g sodium chloride and 200mg thimerosal. In the LH assay, the bovine serum albumin (fraction V; Sigma Ltd.) was added at 1g/l of buffer (0.1% BSA-GAD), and at 5g/l (0.5% BSA-GAD) for the FSH assay. These buffers were stored at 4°C, and fresh batches were prepared each week.

The steroid assay buffer (PBS-Gel) was as for the BSA-GAD, but contained 2g swine skin gelatin (300 bloom; Sigma Ltd.) in place of the bovine serum albumin. The gelatin was dissolved in 400ml of the buffer at 50°C before being added to the remainder of the buffer. After being well mixed, the PBS-Gel was filtered and stored at 4°C. Fresh batches were prepared each week.

2.3.2 Luteinising Hormone

The following assay was developed using an antiserum donated by Dr. R.B. Staigmiller (USDA, Miles City), which was raised in rabbits against a highly purified bovine LH preparation, and affinity purified on Sephadex 4B (Pharmacia Ltd., Milton Keynes). When used at a final dilution of 1:80 000 (200μl of 1:20 000 in an assay volume of 800μl) the antibody bound 35-55% labelled LH; this dilution was used throughout. The assay standard was USDA-bLH-B5, biopotency 2.1 x NIH-LH-B9.

Bovine LH (USDA-bFSH-I1) was radioiodinated after the chloramine-T method of Greenwood, Hunter and Glover (1963). A 5ug aliquot of LH in 30μl 0.5M sodium phosphate, pH 7.4, was iodinated in the presence of 1mCi Na 125I (Amersham International plc, Bucks.) and 20μl chloramine-T (20μg in 0.05M sodium phosphate; Fisons Scientific Apparatus Ltd., Loughborough) followed immediately by 100μl sodium metabisulphite (2.4mg/ml 0.05M phosphate) and 200μl potassium iodide (1% in 0.1% BSA-GAD). The labelled LH was separated from the reaction mixture by column chromatography on Sephadex G50 with the assay buffer.

The final assay procedure was as follows. Standard solutions and serum/plasma samples were made up to 500μl with 0.1% BSA-GAD into 12 x 75mm polyethylene assay tubes and 200μl of antiserum added. The assay tubes were incubated at 4°C for 72 hours before the addition of approximately 20 000 counts per 100 seconds 125I-LH in 100μl and a further incubation at 4°C for 72 hours. The antibody-bound and free label fractions were separated by the addition of 100μl 1:400 normal rabbit serum and 200μl 1:45 donkey anti-rabbit serum (Scottish Antibody Production Unit, Carluke;

SAPU), overnight incubation at 4°C, the addition of 1ml 0.1% BSA-GAD and then centrifugation at 2000 g for 30 minutes at 4°C. The activity in the bound fraction was counted on a Riagamma counter (LKB Ltd., S.Croydon) after pouring and aspiration of the supernatant.

The standard curve was found to be highly reproducible, and had a useful working range of 0.05 to 2.0ng/tube (Figure 2.1). The specificity of the antiserum was assessed against various bovine pituitary hormones. The cross-reactivities were 5.5% for thyroid stimulating hormone (USDA-bTSH-I1), 0.6% for FSH (USDA-bFSH-B1) and <0.1% for growth hormone (USDA-bGH-B1) and prolactin (USDA-bPRL-B1); Figure 2.2). The apparent high cross-reactivity with TSH shown by the antiserum was possibly caused by contamination of the TSH with immunologically active LH. Kelly, Bedirian, Baker and Friesen (1973) have shown that following a thyrotrophin releasing hormone (TRH) injection, serum TSH concentrations rise to a peak after approximately 10 minutes and remain high for approximately 30 minutes. Figure 2.3 shows the serum LH concentrations in five ovariectomised, oestradiol implanted heifers before and after an intramuscular injection of 200μg TRH (Protirelin; Roche Products Ltd., Welwyn Garden City); it is clear that endogenous TSH is not cross-reacting in this assay. Serum samples from ovariectomised and cows post partum were assayed at various dilutions; all exhibited parallelism with the standard curve (Figure 2.4). The accuracy of the assay was assessed by assaying one standard curve in the presence of 300μl serum from a cow post partum; after adjustment for the LH present in the sample, the standard curve was seen to be unaffected by the presence of serum (Figure 2.5). The mean recovery of standard LH from serum over the range 0.15 to 0.75ng/tube was

95.3 ± 7.6%. The sensitivity and coefficients of variation are given for each series of assays in the relevant experimental chapters.

2.3.3 Follicle Stimulating Hormone

FSH was measured by the direct radioimmunoassay of Webb, Lamming, Haynes and Foxcroft (1980) with modifications. Ovine FSH (NIAMDD-oFSH-I1) was iodinated by the lactoperoxidase method of Thorrel and Johansson (1971).

Standards (USDA-bFSH-B1, biopotency 1.7 x NIH-FSH-B1) and serum/plasma samples were made up to 125μl in 0.5% BSA-GAD and incubated with 50μl of a 1:1500 dilution of the antiserum (M94; McNeilly, McNeilly, Walton and Cunningham, 1976) in 1:400 normal rabbit serum (SAPU) for 48 hours at 4°C. The label was added to give 20 000 counts per 100 seconds in 50ul, and the reaction mixture incubated for a further 48 hours at 4°C. Bound and free fractions were separated by the addition of 100μl of a 1:30 dilution of donkey anti-rabbit serum (SAPU), incorporating 1:10 of 0.1M EDTA, and incubation at 4°C for 24 hours. After the addition of 1.5ml assay buffer, all tubes were centrifuged at 2000 g at 4°C for 30 minutes, and the pellet counted for activity on a Riagamma counter (LKB Ltd.).

The specificity of the M94 antiserum has been extensively examined by McNeilly et al (1976) and Webb et al (1980). The sensitivity and coefficients of variation are given for each series of assays in the relevant experimental chapters.

FIGURE 2.1

Composite standard curve for the LH assay. Each point represents the mean (±s.e.m.) from 30 assays.

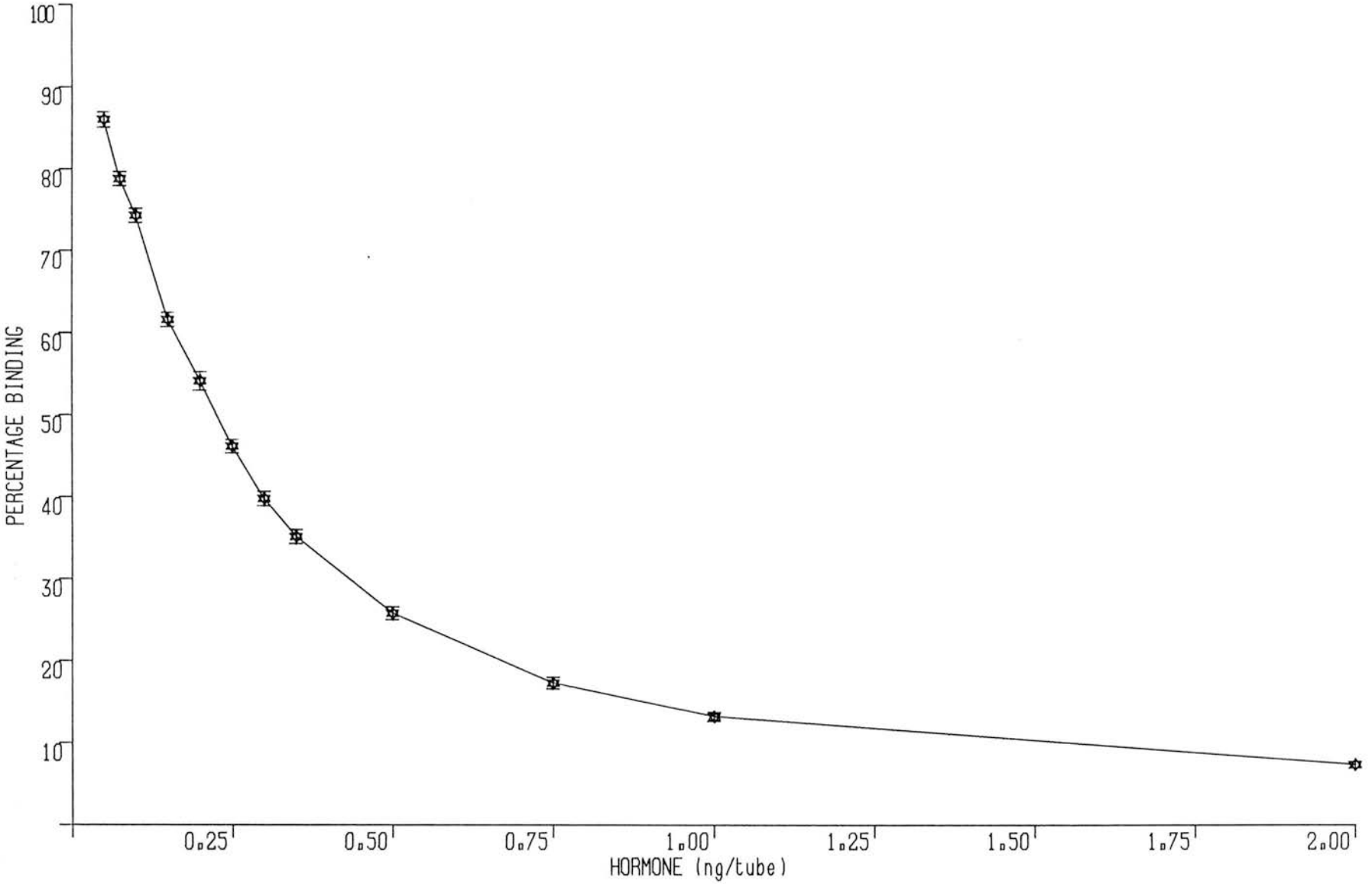

100
90
80
70
60
50
40
30
20
10
PERCENTAGE BINDING
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
HORMONE (ng/tube)

FIGURE 2.2

Specificity of the LH antiserum. The standard curve (————) was tested against bovine FSH (— — —), TSH (------), GH (——---) and prolactin (—— ——).

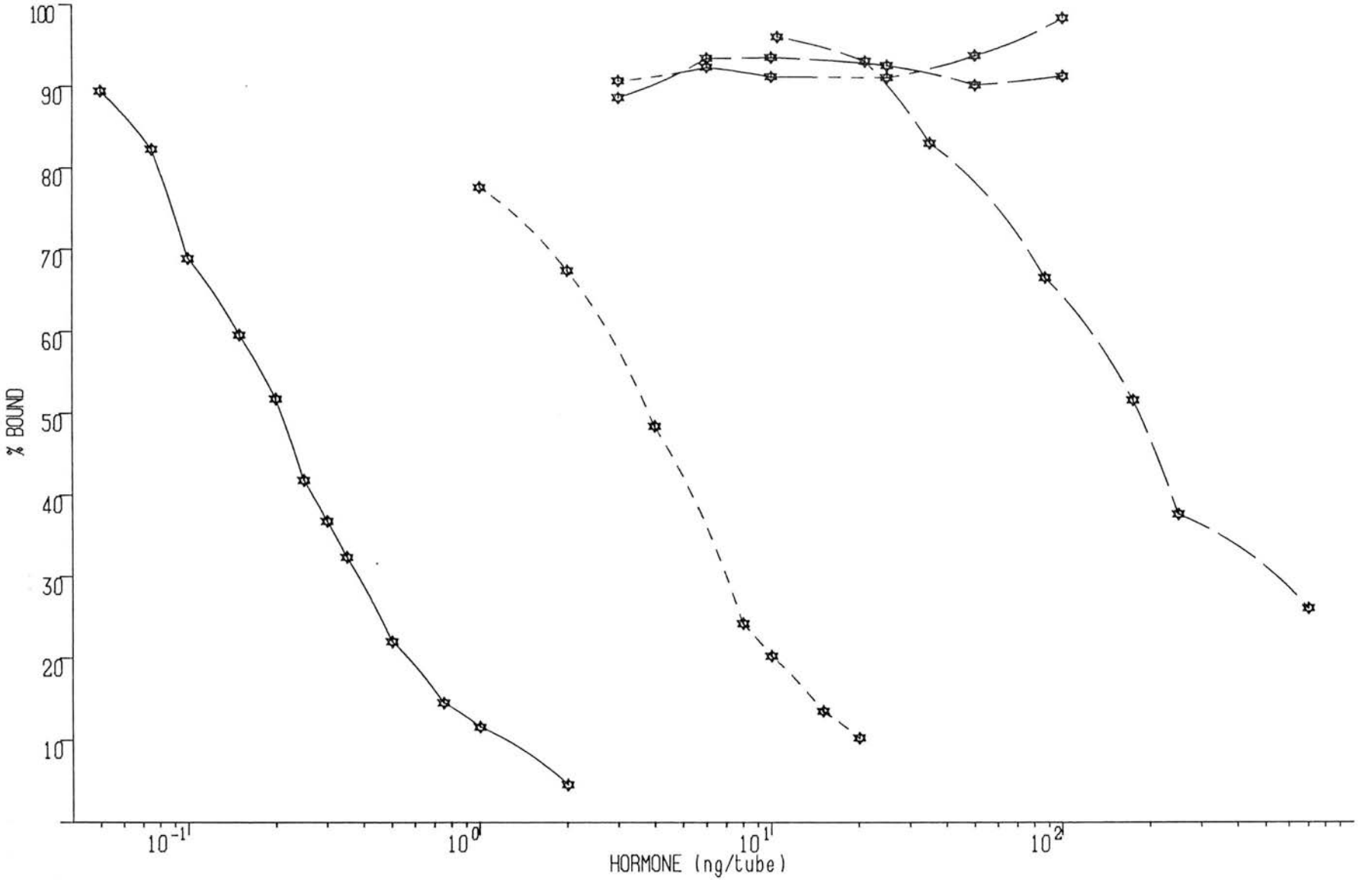

100
90
80
70
60
50
40
30
20
10
% BOUND
10^{-1}
10^{0}
10^{1}
10^{2}
HORMONE (ng/tube)

FIGURE 2.3

LH concentrations in the sera of five ovariectomised, oestradiol implanted heifers, following a bolus injection of TRH.

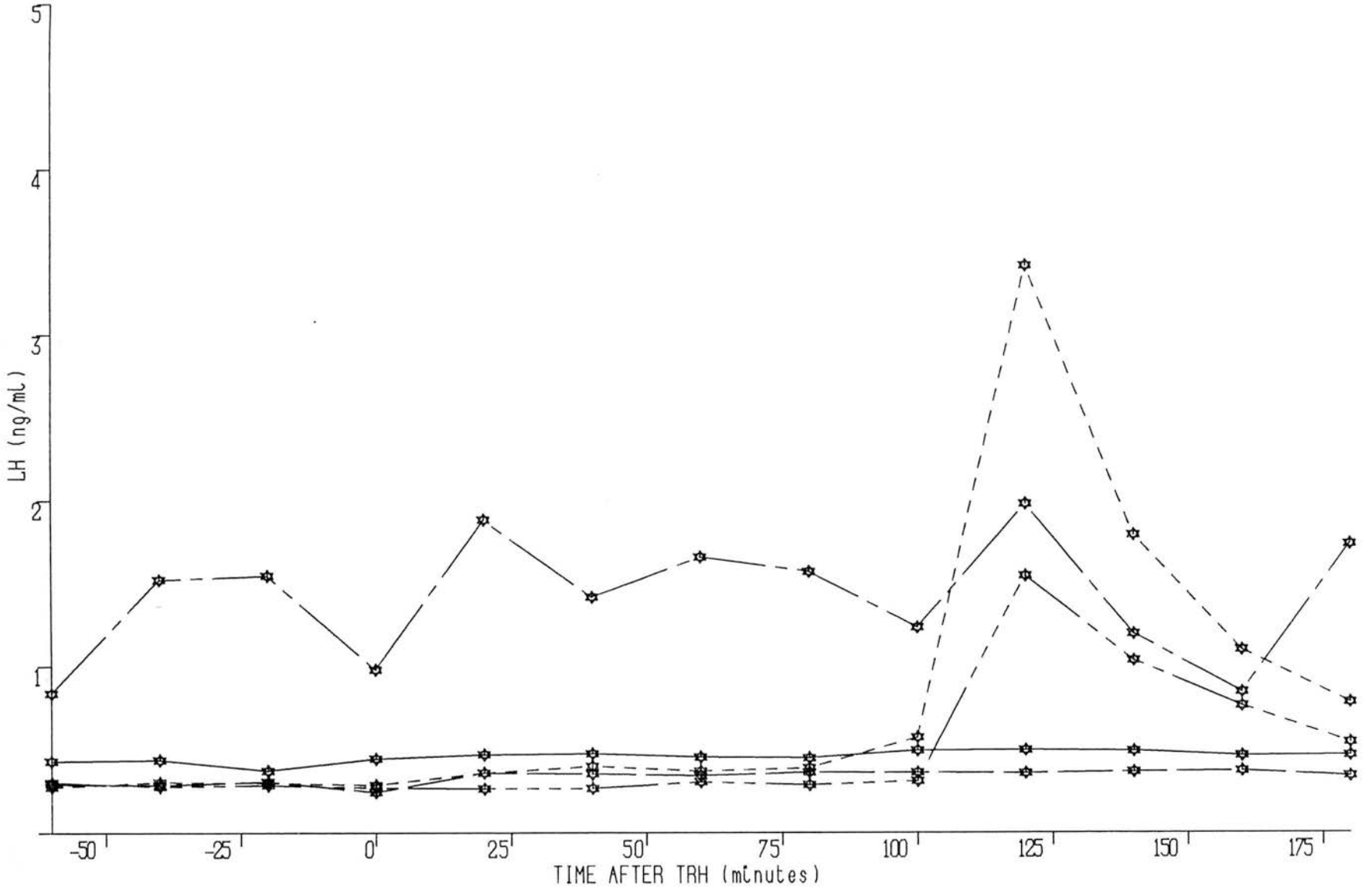

5
4
3
2
1
LH (ng/mL)
-50
-25
0
25
50
75
100
125
150
175
TIME AFTER TRH (minutes)

FIGURE 2.4

Test of parallelism of the LH standard curve (black) with sera from ovariectomised heifers (red) and cows post partum (green).

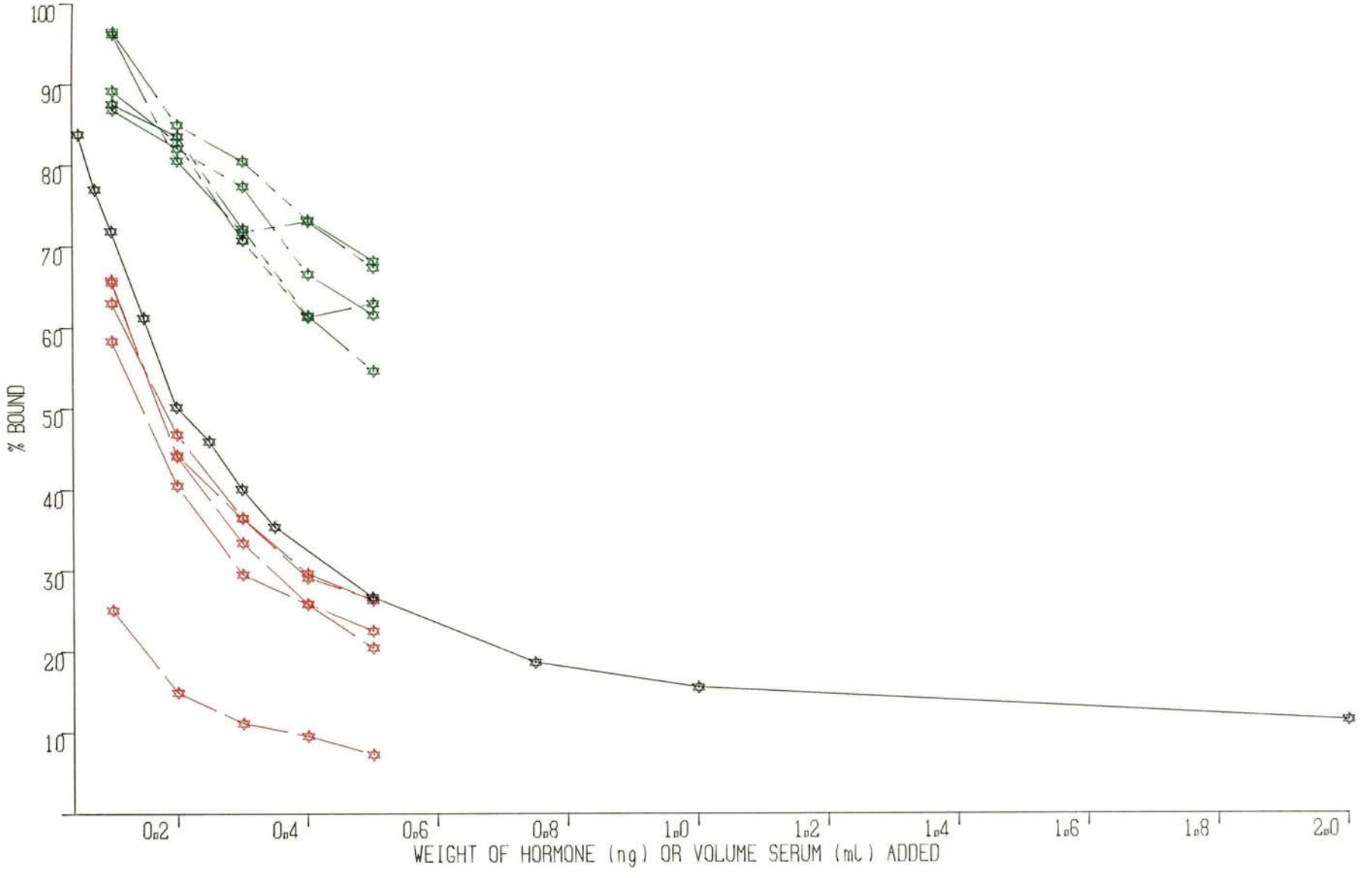
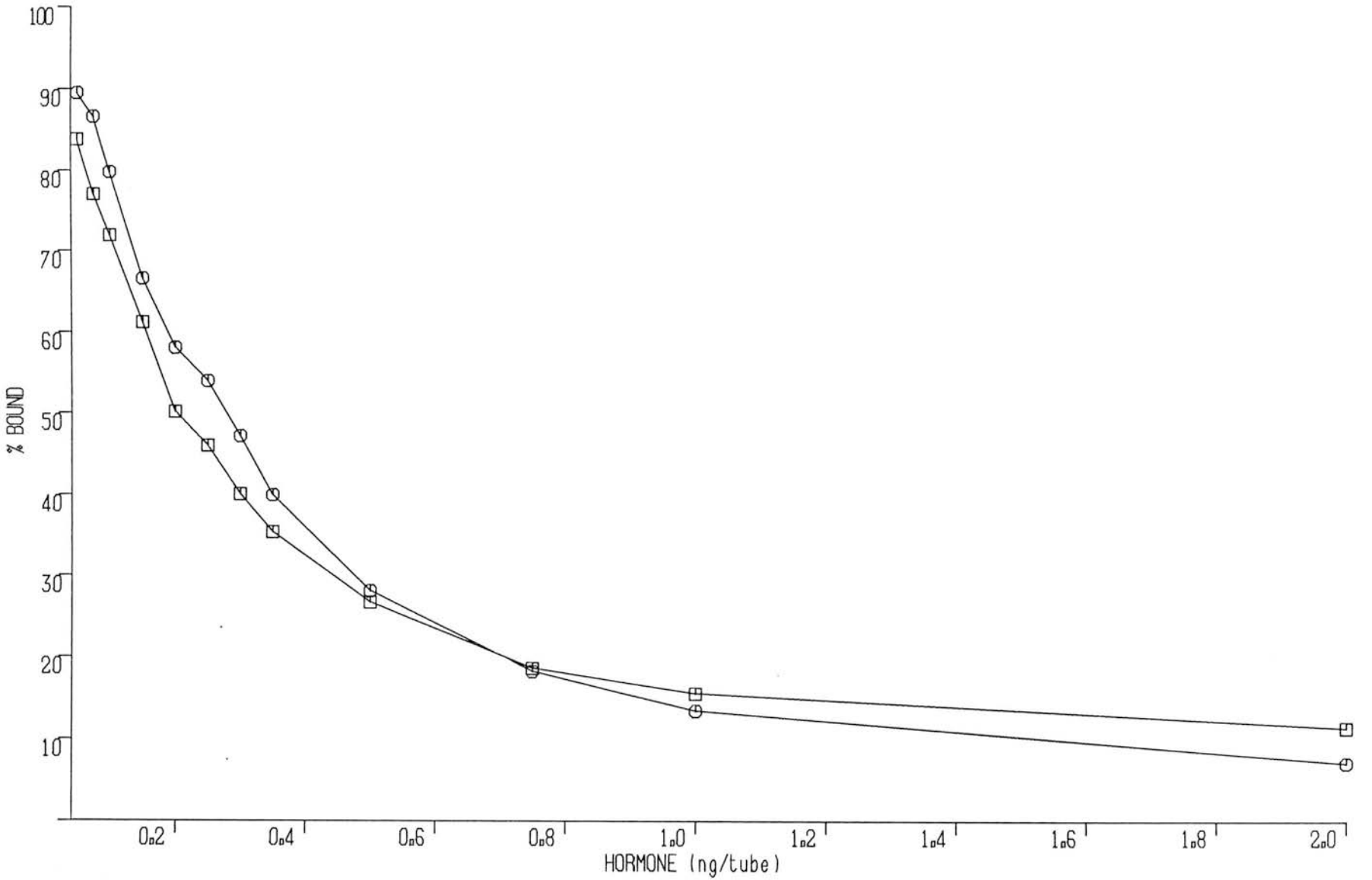

100
90
80
70
60
50
40
30
20
10
% BOUND
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
WEIGHT OF HORMONE (ng) OR VOLUME SERUM (mL) ADDED

FIGURE 2.5

Effect of serum (○) on the LH standard curve (□).


100
90
80
70
60
50
40
30
20
10
% BOUND
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
HORMONE (ng/tube)

2.3.4 Progesterone

Progesterone assays were being routinely performed on plasma samples in the Department, and has been previously validated (Corrie, Ratcliffe & Macpherson, 1982; Ashworth, 1985); the procedure was as follows.

Extraction procedure. Five hundred microlitre aliquots of serum or plasma samples, quality control samples and water were added to glass culture tubes containing 10μl 1,2,6,7-3H-progesterone, approximately 1300 counts per 100 seconds (Amersham International plc); these tubes were mixed and allowed to incubate at room temperature for 30 minutes. Ten parts petroleum ether (40°C-60°C; Fisons Scientific Apparatus Ltd., Loughborough) to one part sample (thus 5ml ether to 500μl sample) was added to the tubes, and mixed for 15 minutes. The tubes were then placed in a dry ice/methanol bath to a depth of approximately 2cm. Once the aqueous phase had frozen, the ether was decanted into glass boiling tubes (16 x 125mm) and dried down on a Buchler Vortex evaporator under vacuum. The steroid extract was then reconstituted into 1.8ml PBS-Gel by mixing at 40°C for 40-50 minutes.

Estimation of extraction recovery. To estimate the extraction efficiency, 500μl of the reconstitute was placed into a plastic minivial (LKB Ltd) and 4ml scintillant (Optiphase X; Fisons Scientific Apparatus) added. An assessment of the activity in 10μl of recovery label was made by mixing 10μl 3H-progesterone, 500μl PBS-Gel and 4ml Optiphase X in minivials, and the background activity assessed by mixing 500μl PBS-Gel and 4ml Optiphase X alone. All vials were counted in a Rackbeta (LKB Ltd.) counter for 300 seconds. The extraction efficiency was calculated for each sample

with the formula (after correction for background activity)

counts X (buffer volume/sample volume)

(usually 1.8/0.5ml =3.6)

and expressed as a percentage of the mean total activity.

Radioimmunoassay. Five hundred microlitre aliquots of the reconstitute were taken in duplicate for radioimmunoassay using the following procedure.

The antiserum was raised in rabbits against progesterone-11α-hemisuccinate-bovine serum albumin by the method of Dighe & Hunter (1974) and provided by Dr. J.E.T. Corrie (MRC Immunoassay Team, Edinburgh). Standards, prepared from a stock solution of progesterone (Sigma) in 'aristar' ethanol (BDH Chemicals Ltd., Glasgow), were made up to 500μl with PBS-Gel in 12 x 75mm glass assay tubes and 200μl 1:8000 antiserum added, followed by 100μl 125I-progesterone-11α-glucuronide-tyramine (prepared by the method of Corrie, Hunter & Macpherson, 1981; provided by the MRC Immunoassay Team) to give approximately 20000 counts/ 100 seconds per tube, and incubated at room temperature for two hours.

Separation of bound and free counts was effected by the addition of 100μl of a 1:35 dilution of donkey anti-rabbit serum (SAPU) followed by 100μl of a 1:300 dilution of normal rabbit serum (SAPU) and overnight incubation at 4°C. Immediately prior to centrifugation, each tube received 1ml PBS-Gel at 4°C; after centrifugation at 2000g at 4°C for 30 minutes, the supernatant was decanted and the pellet assessed for radioactivity on a Riagamma counter (LKB Ltd). The resulting sample potencies were corrected for recovery losses, and expressed as ng/ml.

The specificity of the antiserum is summarised in Table 2.1. The quality control samples consisted of a water 'blank', follicular phase cow serum, serum from a cow post partum, and a mixture of these two sera to give potencies of approximately 0.2, 1.5 and 3.0ng/ml. The potency estimates of the water 'blanks' were below the limit of sensitivity of the assay. The coefficients of variation were calculated from these control samples as described below, and, with the assay sensitivity, are given in the relevant experimental chapter.

2.3.5 Oestradiol

Oestradiol concentrations were determined in culture media by direct radioimmunoassay, but plasma oestradiol was extracted by affinity chromatography before assay. The preparation of the label and antiserum, and the validation of the assay procedure have been reported in detail elsewhere (Webb, Baxter, McBride, Nordblom & Shaw, 1985b); the specificity of the antiserum is summarised in Table 2.2.

Briefly, the oestradiol antiserum (R48) was raised in rabbits against oestradiol-11β-succinyl-bovine serum albumin. The radiolabel, 17β-oestradiol-11α-tyrosinemethylester, was iodinated in the presence of chloramine-T by the method of Hunter, Nars & Rutherford (1975); the label was separated from the reaction mixture by column chromatography on Sephadex G25, and eluted under pressure with 0.05M sodium phosphate buffer (pH 10.5).

Extraction procedure. The oestradiol antiserum was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the

TABLE 2.1

Specificity of the progesterone antiserun.

Steroid tested	Percentage cross-reactivity
Progesterone	100.0
11α-Hydroxy-progesterone	80.6
17α-Hydroxy-progesterone	4.6
20α-Hydroxy-4-pregnene-3-one	1.4
Corticosterone	0.6
Oestrone	0.4
16α-Hydroxy-progesterone	0.9
Androsterone	0.6
17α-Oestriol	0.5
5α-Androstene-3,17-dione	0.07
*Androst-4-ene-3,17-dione	0.025
*Cortisol	0.005
*Oestradiol	<0.001
*3β-Hydroxypregn-5-en-20-one	0.03
*20α-Hydroxypregn-4-en-3-one	1.2
*5α-Pregnane-3,20-dione	13.1
*Testosterone	0.01

Data from Ashworth (1985), except those marked *, from Corrie, Ratcliffe and Macpherson (1982).

manufacturers instructions, to give 9g (dry weight) Sepharose suspended in 75ml phosphate buffered saline (0.05M, pH 7.5, 0.01% thimerosal). One hundred microlitres of this suspension were added to screw-capped glass culture tubes (16 x 125mm) containing 3ml plasma sample, 10μl of 1000 counts/100 seconds 2,4,6,7,16,17-3H-oestradiol-17β (Amersham International plc) and 10ml water. The samples were mixed end-over-end overnight at 4°C.

The chromatography columns (10 x 120mm soda glass with glass sinter discs, porosity 1; Schott Glass, UK) were washed with 3ml 90% methanol followed by three rinses of 7ml water, this cycle being repeated at least twice. The mixed contents of the culture tubes were then tipped directly into the columns; the Sepharose residues in the culture tubes were rinsed out with 7ml water and applied to the respective columns. Each column was washed through with 21ml water under gentle pressure and the washings discarded. Sepharose-bound oestradiol was then eluted into 16 x 125mm glass tubes with 3ml 90% methanol, again under positive pressure. The eluate was evaporated and reconstituted as for the progesterone assay above. Five hundred microlitre aliquots of each sample extract were used for estimating extraction efficiency as described for progesterone.

Radioimmunoassay. Duplicate 500μl aliquots of the sample reconstitute were added to glass assay tubes with 200μl 1:40000 antiserum and 100μl 125I-oestradiol (approx. 20000 counts/100 seconds), mixed, and incubated at room temperature for two hours. Standards were prepared from a stock solution of oestradiol-17β (Sigma) in 'aristar' ethanol (BDH) to cover the range 0.5 to 48pg/tube. Separation of bound and free label was achieved by the addition of 100μl 1:40 donkey anti-rabbit serum (SAPU) and 100μl

1:400 normal rabbit serum (SAPU) and incubation at 4°C for 24 hours. After centrifugation and aspiration of the supernatant, the tubes were assessed for radioactivity on a Riagamma counter (LKB Ltd) and the potency estimates corrected for recovery losses.

To ensure that this assay was capable of detecting peripheral oestradiol in the cow, blood samples were taken daily from untreated heifers for one oestrous cycle, and the plasma assayed. The resultant profiles are shown in Figure 2.6. The quality control samples consisted of a water 'blank', ovariectomised heifer plasma, and oestradiol incorporated into ovariectomised heifer plasma to give 5.0, 10.0 and 20.0pg/ml. These control plasmas were used to calculate the coefficients of variation, and, with the assay sensitivity, are given in the relevant experimental chapters.

2.3.6 Assay Statistics

Assay sensitivities (defined as twice the standard deviation of blank values) and coefficients of variation for each series of assays are given in the relevant experimental chapters. The inter-assay coefficient of variation was calculated from potency estimates of the quality control sera in each assay, according to the formula

$$\frac{SD}{\bar{x}} \times 100$$

TABLE 2.2

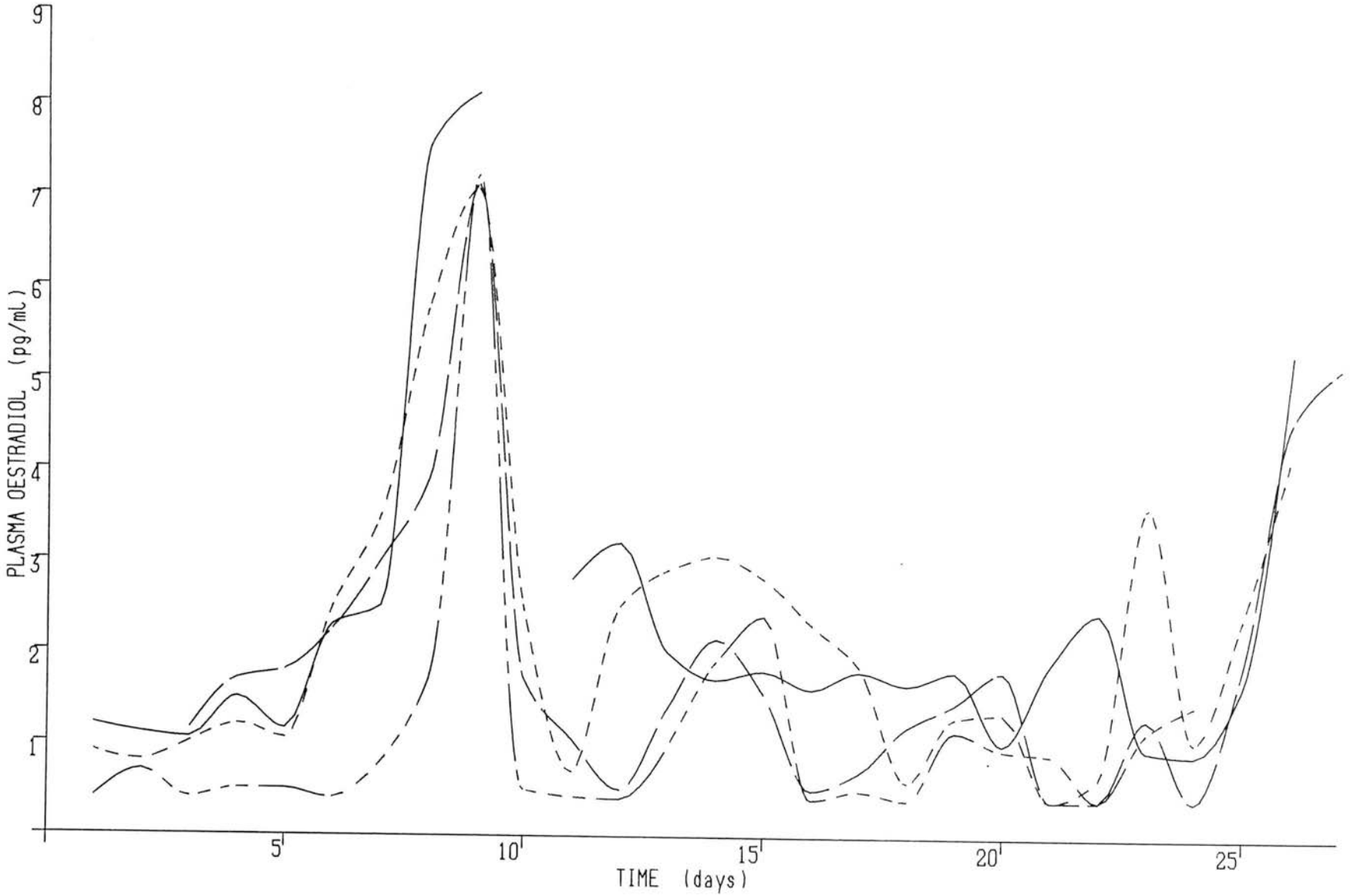
Specificity of the oestradiol (R48) antiserun.

Steroid tested	Percentage cross-reactivity
Oestradiol-17β	100.0
Oestrone	16.0
Oestriol	3.0
Testosterone	1.2
5α-Dihydrotestosterone	0.1
Androsterone	0.01
Androstenedione	0.1
Progesterone	0.01
5-Androstan-3β,17β-diol	<1.0
Cortisol	<1.0
Corticosterone	<1.0
Etiocholan-3α-ol-17-one	<1.0
5-Pregnen-16α-ol-3,20-dione	<1.0
4-Pregnen-17α-ol-3,20-dione	<1.0
4-Pregnen-20α-ol-3-one	<1.0
4-Pregnen-16α-ol-3,20-dione	<1.0

Data from Webb, Baxter, McBride, Nordblom and Shaw (1985b)

FIGURE 2.6

Plasma oestradiol profiles of four heifers sampled daily throughout the oestrous cycle. Profiles were adjusted for the time of oestrus (shown on the tenth day of sampling).


PLASMA OESTRADIOL (pg/mL)
9
8
7
6
5
4
3
2
1
5
10
15
20
25
TIME (days)

where SD is the standard deviation of the quality control potencies and x is the mean quality control potency. The intra-assay coefficient of variation for the gonadotrophin assays was estimated from one assay in each series with 20 randomly chosen sample potency estimates. The difference between the upper and lower 95% confidence limits for each sample is divided by four to estimate the standard error (se) of the potencies. The standard deviation (sd) of these potencies is then calculated by the formula

$$sd = se \times \sqrt{n}$$

where n = number of replicates. An estimate of the overall standard deviation (Tsd) is given by

$$Tsd = \sqrt{\frac{\sum sd^2}{2N-1}}$$

where N = number of samples. The coefficient is then given by

$$\frac{Tsd}{\bar{x}} \times 100$$

In the steroid assays the intra-assay coefficient of variation was calculated either as for the gonadotrophins, or by using the formula for the inter-assay coefficient on the potencies of several sets of control plasmas which were interspersed through each assay.

2.4 IMMUNISATIONS

Several of the experimental chapters involved actively immunising heifers against steroid and protein hormones. To avoid repetition, the preparation of the antigens and emulsion are described here; refer to the relevant chapters for details and protocols.

2.4.1 Preparation of the Inhibin Antigens

The inhibin antigens were prepared by the method of Jansen et al. (1981). Bovine, ovine and porcine ovaries were collected on ice from a local slaughterhouse, and follicular fluid aspirated aseptically from all follicles that were greater than 2mm in diameter and that were not obviously cystic, and centrifuged to remove cell debris. Equine follicular fluid was obtained from the Institute National de la Recherche Agronomique, Nouzilly. The follicular fluids were incubated with 1mg/ml activated charcoal (Hopkin & Williams Ltd., Chadwell Heath, Essex) and 0.1mg/ml dextran T-70 (Pharmacia) for one hour at room temperature or overnight at 4°C to greatly reduce the steroid content of the fluid, and were then applied to a chromatography column containing 10g Red Sepharose CL-4B (Pharmacia; bed height 23cm) at 4°C. The column was equilibrated, and the sample applied with a 25mM tris(hydroxymethyl)aminomethane (Tris; Sigma) buffer containing 0.35M potassium chloride and 1M urea. The immunogen was eluted off the column with a 25mM Tris, 1M urea and 1.2M potassium chloride buffer. The column was regenerated between runs with 1.5M sodium

chloride with 0.1% thimerosal.

With the exception of the single batch used in Chapter 6 (ovine-I, supplied by Dr. T. O'Shea, University of New England, Australia), all preparations (i.e., ovine-II, bovine, equine and porcine) were concentrated and dialysed against sterile saline in a stirred ultrafiltration cell on a membrane with a molecular weight cut-off of 5kDa (YM5; Amicon Ltd., Stonehouse). Column eluates were pooled to form uniform batches and then sterilised by exposure to 250Gy ($\underline{=}$ 25000 rads) from a 137-Caesium source. The total protein content of these batches were determined by the method of Lowry, Rosebrough, Farr and Randall (1951). The single batch of material used in Chapter 6 was not gamma-sterilised, but was checked for sterility by streaking onto a Chocolate Horse Blood Agar plate (Gibco Ltd, Paisley); it was found to be devoid of live bacteria.

2.4.2 Preparation of the Testosterone Antigen

The testosterone antigen was provided for these studies as testosterone-3-carboxymethyloxime-ovalbumin (T-3CMO; B.A.Morris, Guildhay Antisera, University of Surrey).

2.4.3 Preparation of the Emulsion

All immunisations were carried out with a water-in-oil emulsion (Herbert, 1978) with equal volumes of oil and aqueous phases. The mineral oil, Non-Ulcerative Freund's Adjuvant (NUFA; Guildhay Antisera, University of Surrey) was emulsified with sterile saline

into which the antigens and immunostimulant were incorporated. The immunostimulant was Corynebacterium parvum (Wellcome Biotechnology Ltd., Beckenham; Adlam, Broughton and Scott, 1972; Halpern, Fray, Crepin, Platica, Lorinet, Rabourdin, Sparros and Isac, 1973), which was being used in this context for the first time. It was of importance to identify an adjuvant for use in cattle that did not provoke the necrotic lesions commonly encountered when using Mycobacterium tuberculosis in Freund's Complete Adjuvant, and that would not produce false results in routine tuberculosis tests. The emulsion was always injected intramuscularly over several sites. Additionally, where specified, animals also received a subcutaneous injection of Bordetella pertussis (Wellcome Foundation Ltd., London).

2.5 GENERAL STATISTICS

Arithmetic or geometric means are presented with their standard errors (s.e.m.) as specified in each chapter. Generally, endocrine profiles are expressed as geometric means, all other data as arithmetic means. Differences between means were tested by Student's t test or analysis of variance for (with correction for multiple comparisons) normally distributed data and by the Mann-Whitney ranked test for non-parametric data.

LH profiles were examined for the occurrence of pulses by two methods. Firstly, data which showed a low frequency, high amplitude pulse profile were examined by a programme which defined a pulse as a peak in concentration whose lower 95% confidence limit was greater than twice the difference between the previous nadir and its upper

95% confidence limit added to the value of the nadir (Webb et al., 1985a). These results were expressed as the number of pulses per 12 hours. Secondly, data which showed a high frequency, low amplitude pulse profile were subject to time series analysis to identify the major episodic rhythm, and these results were expressed as the pulse interval in minutes. The validity of using these two techniques has been discussed in full by McLeod and Craigon (1985).

Simple statistics were calculated on the Minitab statistical package (Pennsylvania State University, USA) and more complex calculations were performed by the Genstat programme (Rothamsted Experimental Station).

CHAPTER THREE

ACTIVE IMMUNISATION OF THE COW AGAINST TESTOSTERONE

3.1 INTRODUCTION

The use of immunological techniques to increase prolificacy in sheep has been widely explored (see Chapter One). Active immunisation against oestrone, androstenedione and testosterone have been shown to increase ovulation and lambing rates (Scaramuzzi et al., 1977; Cox et al., 1982). By contrast, immunisation against oestradiol-17β, oestrone and testosterone, have been reported to result in a high incidence of blocked or delayed oestrous cycles (Rawlings et al., 1978; Martensz and Scaramuzzi, 1979). Attempts have been made to immunise cattle against steroids to induce twinning, but with less success than in sheep. Active immunisation against oestradiol-17β has been shown either to disrupt or to have no effect on oestrous cycles (Martin et al., 1978; Wise and Schanbacher, 1983), whilst increased ovulation and calving rates have been reported following immunisation against androstenedione (Wise and Schanbacher, 1983).

The aim of this study was to assess the effects of actively immunising heifers against a testosterone conjugate on ovulation rate, oestrous cycle length and peripheral serum hormone concentrations. After this experiment had started, two preliminary reports appeared in the literature describing experiments in which cattle were immunised against testosterone; Sreenan et al. (1983) increased the incidence of anoestrus, but with no double ovulations, whereas D'Occhio et al. (1986) reported 2 of 7 immunised heifers showed double ovulations without displaying anoestrus.

3.2.1 Protocol

Seventeen Charolais and Simmental cross yearling heifers were divided into two treatment groups of four and a control group of nine animals. The treatment animals were immunised by injection with 8mg T-3CMO (see Chapter Two) dissolved in adjuvant A (1.5ml NUFA plus 1.5ml sterile saline; group A) or adjuvant B (1.5ml NUFA, 0.5ml sterile saline and 1.0ml *C. parvum*; group B) given intramuscularly at four sites, in conjunction with 2.5ml *Bord. pertussis* injected subcutaneously in the brisket. The control animals were not injected.

After the priming injection, both treatment groups were given two booster injections at four month intervals; the first booster injection was of the same composition as the priming injection, but the second booster injection was devoid of *C. parvum* and *Bord. pertussis*.

Jugular blood samples were taken by vacutainer three times weekly after the second booster injection for antibody titre, serum progesterone and tonic LH determinations. In addition, control animals were sampled from cannulae every 10 minutes for 12 hours for FSH and pulsatile LH profile determinations during the luteal (Day 9-12) and follicular (Day 17-19) phases of the oestrous cycle. Immunised animals which showed regular oestrous cycles were sampled as for the control heifers, and those which appeared to be anoestrous were sampled for one period of 8 hours.

To compare the endocrine effects of the current partial immunocastration (see Results) with those of total physical castration, three Hereford-Friesian crossbred heifers which had been ovariectomised for 8 months, were cannulated and blood sampled every 10 min for 6 hours.

Observations for oestrus and ovulation rate (by mid-ventral laparoscopy) were made after the second booster injection.

3.2.2 Antibody Characterisation

Antibody titres were determined by the addition of 0.1ml 1:50000 dilution (final dilution of 1:150000 in 300μl reaction volume) of each antiserum to 0.1ml PBS-Gel and 0.1ml of a tritiated testosterone solution (Amersham International; specific activity 93Ci/mmol; mean mass added per tube, 77.1 $\pm$ 2.8pg) in PBS-Gel. The reaction mixture was incubated overnight at 4°C, then 1ml dextran-charcoal suspension (Pharmacia; 25mg dextran T-70 : 250mg charcoal in 100ml PBS-Gel) was added and all tubes incubated for a further 30 min at 4°C before centrifugation at 2000g for 20 min. The supernatant containing antiserum-bound testosterone was decanted into 3ml Fisosorb 4, mixed and the activity counted. The titre was expressed as the percentage of added label bound by the dilution of antiserum present. Two types of serum sample were utilised in this assay for quality control. The negative control sample consisted of a serum sample from a non-immunised control heifer, added at initial dilutions of 1:1000, 1:5000 and 1:10000. The positive control sample consisted of a pooled serum sample from a sheep previously immunised against testosterone, and was used at initial dilutions of

1:10000 and 1:100000. The inter- and intra-assay coefficients of variation, based on five control duplicates in each of eight assays were 13.0% and 5.5%, respectively.

The same procedure was adopted, with minor modifications, to assess the specificity (serum used at a dilution of 1:7000, final dilution 1:21000) and affinity (serum used at a dilution of 1:4000, final dilution 1:12000; unlabelled testosterone added to cover the range 5.0 to 250pg/tube; 30.7pg label added per tube) of the antisera.

3.2.3 Hormone Assays

The sensitivity of the LH assay was 0.07 $\pm$ 0.01ng/tube, calculated over six assays, and the inter- and intra-assay (20 duplicate samples) coefficients of variation were both 3.4%.

All FSH determinations were completed in one assay, with a sensitivity of 0.4ng/tube, and intra-assay coefficient of variation of 2.2%.

The extraction phase of the progesterone assay was modified by the addition of 100μl 0.1M hydrochloric acid to the petroleum ether to denature protein-steroid complexes (Scaramuzzi et al., 1980a). The extraction efficiency was 70% and the sensitivity of the assay was 0.12ng/ml. The inter- and intra-assay coefficients of variation over 4 assays were 14.5 and 15.8%, and 14.8 and 20.8% respectively for pooled serum samples containing 1.5 and 3.0ng/ml.

3.2.4 Statistics

The affinities of the antisera raised against testosterone were calculated by the method of Scatchard (1949) after correction for non-specific binding (Chamness and McGuire, 1975).

3.3 RESULTS

3.3.1 Immunological Response

Of the eight immunised heifers, seven displayed antibody responses after the second booster injection (Table 3.1; Figure 3.1). There were no apparent differences between the adjuvants, so both treatment groups were pooled; the non-responding heifer (number 624, Table 3.1) was discarded from the analysis. No animals developed any visible lesions arising from the use of C. parvum. At the dilutions tested, the antisera were both specific (cross-reactivities of 2.5% for androstenedione and <0.6% for oestradiol-17β, oestrone and progesterone) and of high affinity (mean affinity dissociation constant, Kd = 3.5 $\pm$ 0.4x10[-10]M) and there were no significant differences between the antisera tested. At no time did the titre for the negative controls exceed 1.5% binding of labelled testosterone.

TABLE 3.1

Antibody titres in all treatment animals, and overall mean titres, following the second booster injection. Titres are expressed as percentage binding of added label (77.2pg/tube) at a final antibody dilution of 1:150000, and are given at peak values (within three weeks of boosting), and at 10, 25 and 40 weeks following booster.

	Titre			
	Peak titre	10 weeks	25 weeks	40 weeks
Group A: animals 553	11.8	3.9	14.2	4.9
586	12.9	6.5	3.4	+
590	23.6	8.2	3.0	1.4
624	<0.5	<0.5	<0.5	*
Group B: animals 558	11.1	3.4	2.0	0.8
566	5.7	3.5	1.9	1.1
589	9.7	5.0	4.2	1.5
601	12.4	5.2	4.8	1.9
Treatment mean±s.e.m. $	12.4 ± 2.1^{a}	5.1 ± 0.7^{b}	4.8 ± 1.6^{b}	1.9 ± 0.6^{c}

+ Animal slaughtered 27 weeks after booster

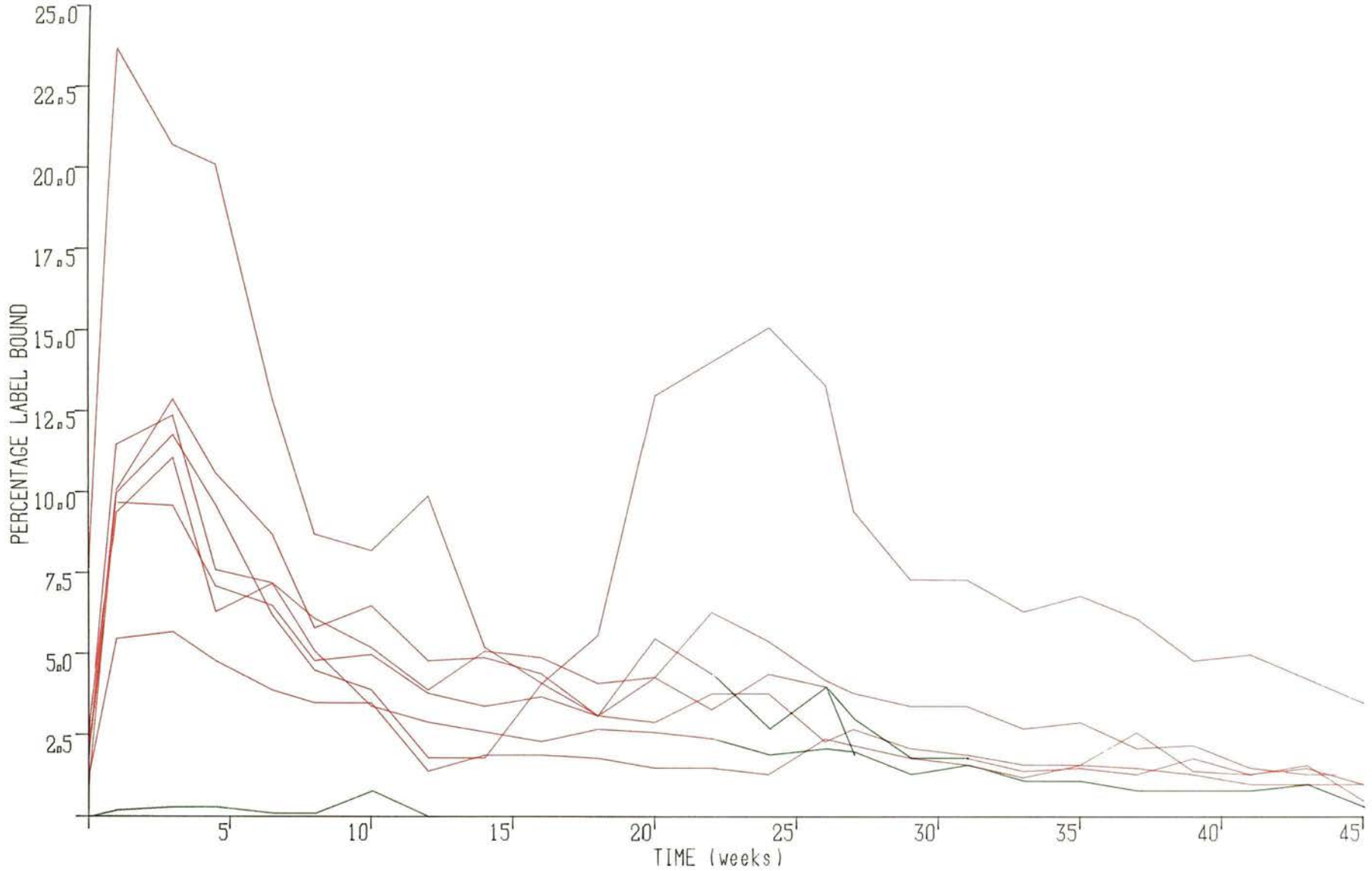
* Animal did not respond to immunisation and was discarded at 27 weeks

$ Means do not include values for heifer 624 (non-responder)

a,b,c Mean values with different superscripts differ ($P<0.05$)

FIGURE 3.1

Testosterone antibody titres in all heifers following the second booster injection. The animals did not display oestrus during the periods when the titres are shown in red, but oestrous activity was observed when the titres are shown in green.


25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
PERCENTAGE LABEL BOUND
5
10
15
20
25
30
35
40
45
TIME (weeks)

3.3.2 Ovarian Responses

Following the second booster injection, all responding heifers ceased to show regular oestrous cycles, and displayed large cystic structures in the ovaries (Figure 3.2). The non-responding heifer continued to show regular oestrous cycles and single ovulations.

Approximately 25 weeks after the second booster injection, three heifers showed some oestrous activity. Heifer 558 gave two consecutive double ovulations, followed by one anovulatory oestrus, and two consecutive cycles with single ovulations; cystic structures were seen on both ovaries at each occasion. This animal was intensively sampled for gonadotrophin profiles following the second double ovulation. Heifer 586 exhibited one double ovulation accompanied by cystic structures, and then two consecutive single ovulations, again in the presence of ovarian cysts. This heifer was left undisturbed for several cycles and then ovariectomised at slaughter. Three mature corpora lutea were seen on one ovary, and the contralateral ovary contained a large fluid filled cyst with a thick luteinised wall and fluid volume of 7.5ml (Figure 3.3). The antibody titres in this fluid, and the fluid from several other follicles, were similar to that in a serum sample also taken at slaughter (Table 3.2). The third heifer, 589, displayed four corpora lutea on one ovary and no cystic structures. This animal became anoestrous again, and laparoscopy revealed the presence of the four ovulations as corpora albicantia, plus one cyst, but no fresh corpora lutea. Oestrus was not seen in this animal for a further three months, after which it was removed from the experiment because of injury. There was no correlation between antibody titre and oestrous activity at 25 weeks after boosting (Table 3.1; Figure

3.1).

The remaining four immunised heifers showed sporadic signs of behavioural oestrus, but all were anovulatory and the ovaries were cystic. All control heifers continued to show regular oestrous activity.

3.3.3 Progesterone Concentrations

Progesterone profiles were examined for a period of six weeks following the second booster injection, and all anoestrous heifers showed very high concentrations (up to 90ng/ml; Figure 3.4) which fluctuated irregularly. All control heifers showed regular cycles of progesterone secretion.

At approximately 25 weeks following the second booster injection, the progesterone profiles from the multiple ovulating heifers (558, 586, 589) showed a marked cyclical activity, although peak concentrations were still abnormally high (16ng/ml, heifer 558; 30ng/ml, heifer 589); at this time no control heifer showed concentrations higher than 6.5ng/ml. The four anoestrous, immunised heifers continued to exhibit irregular patterns of progesterone secretion.

FIGURE 3.2

The ovaries of two anoestrous, immunised heifers, viewed *in situ* after the second booster injection, showing follicular- and luteal-type cystic structures (A & B, repectively).

A

B

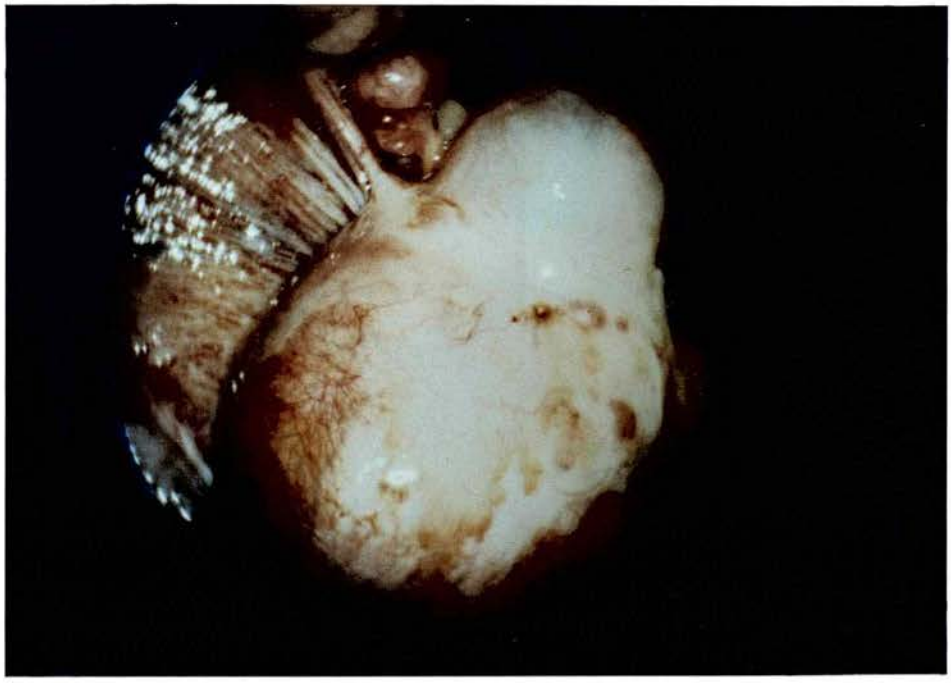


FIGURE 3.3

Ovaries excised from a representative control heifer (A), and from heifer 586 at slaughter (B) and following dissection (C). One ovary from heifer 586 contained three corpora lutea (cl), and the other contained large follicles with volumes of 7.5ml (fi) and 2.0ml (fii). The control heifer shown in this figure was taken from Chapter 4, as no control heifers were ovariectomised in the present study.

A

B

C

TABLE 3.2

Antibody titre, expressed as percentage binding of added label (77.2 pg 3H-testosterone/tube) at final antibody dilutions of 1:75000, 1:150000 and 1:600000, in serum and follicular fluid samples from a cycling, immunised heifer (586). Samples were taken at slaughter, 27 weeks after the second booster injection.

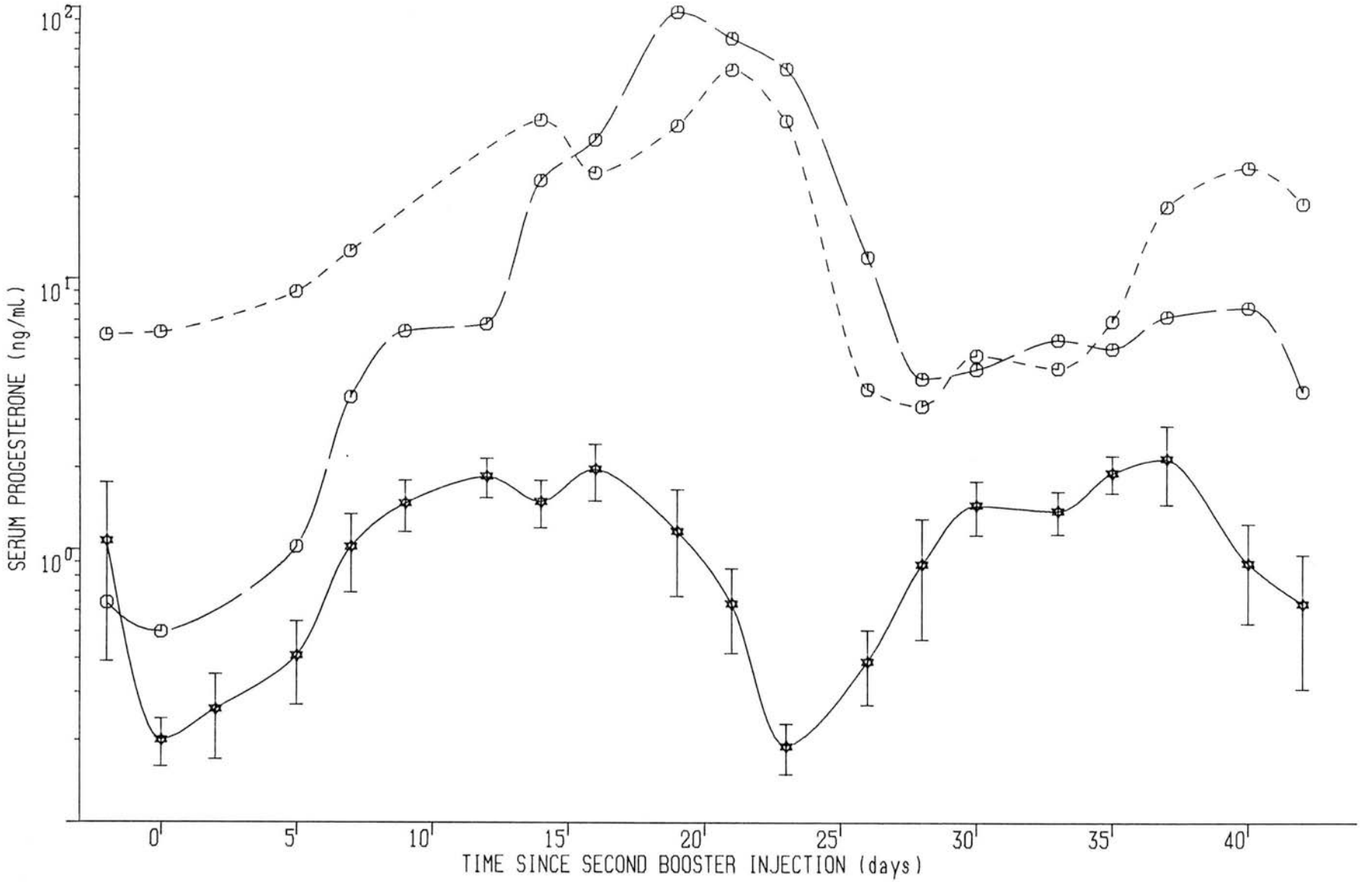
	Serum	Follicular fluid		
Sample dilution		+ Cyst	Large follicle*	Small follicle pool
1: 75 000	20.0	16.2	9.0	16.2
1:150 000	8.9	7.7	3.8	8.6
1:600 000	2.7	2.7	0.9	2.0

+ Cyst volume, 7.5ml. See Figure 3.3

* Follicle volume, 2.0ml.

FIGURE 3.4

Mean (±s.e.m.) progesterone concentrations of six control heifers, adjusted to the time of oestrus, and progesterone profiles from two anoestrous, immunised heifers (586, —— —— ; 601, ------) following the second booster injection.


SERUM PROGESTERONE (ng/mL)
10^2
10^1
10^0
0
5
10
15
20
25
30
35
40
TIME SINCE SECOND BOOSTER INJECTION (days)

3.3.4 Gonadotrophin Concentrations

The mean LH concentrations in the sera of the immunised animals were significantly higher ($P<0.001$) than that of the control heifers (1.01 ± 0.03 and 0.45 ± 0.06ng/ml respectively) just prior to, and did not change following, the second booster injection.

The data from the LH and FSH intensive sampling periods, approximately 25 weeks after the second booster injection, are summarised in Table 3.3. Retrospective consideration of the progesterone data indicated that only one control heifer (603) was in the follicular phase (see Schallenberger *et al.*, 1984) and therefore is considered alone in Table 3.3. The LH profiles for the one immunised heifer showing oestrus at the time of sampling (558), two anoestrous immunised heifers (590 and 601), and the representative control (603) are given in Figure 3.5.

The variation in mean LH concentrations between cows was high in the luteal phase control heifers, and in the anoestrous, immunised heifers ($P<0.01$), although the difference between these groups was also significant ($P<0.05$), despite different apparent concentrations of progesterone (Table 3.4). There was no difference in mean LH concentrations between the anoestrous heifers and the follicular phase control heifer. The cycling, immunised heifer (558) displayed similar mean luteal phase and follicular phase LH concentrations, but these were significantly lower than those of both luteal and follicular phase control animals ($P<0.01$; Table 3.3). The mean LH pulse frequency in the anoestrous, immunised heifers is higher than that in the luteal phase control animals (although a direct comparison cannot be made since the two parameters are not measured in the same units), but not different from that of the

TABLE 3.3

Secretory pattern of LH and FSH in control and immunised heifers. Mean values are expressed as geometric means (±s.e.m.).

Group	No. of heifers	LH			FSH
		Mean (ng/ml)	Pulse frequency (per 12h)	Pulse interval (min)	Mean (ng/ml)
Controls:					
Luteal phase	5	0.59±0.08[a]	1.5±1.2	-	17.1±1.5[wx]
Follicular phase	1	1.03±0.03[b]	-	48	19.5±1.8[w]
Ovariectomised	3	2.32±0.16[d]	-	31.0±1.0[e]	102.9±9.3[y]
Immunised:					
Luteal phase	1	0.37±0.02[c]	3	-	13.6±1.9[xz]
Follicular phase	1	0.37±0.03[c]	5	-	6.9±0.9[v]
Anoestrous	5	0.94±0.09[b]	-	32.1±1.1[e]	11.5±1.3[z]

a,b,c,d,e,v,w,x,y,z Mean values down a column without the same superscript differ ($P<0.05$)

FIGURE 3.5

Plasma LH profiles during the luteal (A) and follicular (B) phase of the representative control heifer, and of two anoestrous, immunised heifers (C, 553; D, 590), approximately 25 - 30 weeks after the second booster injection. Samples were taken every 10 min.

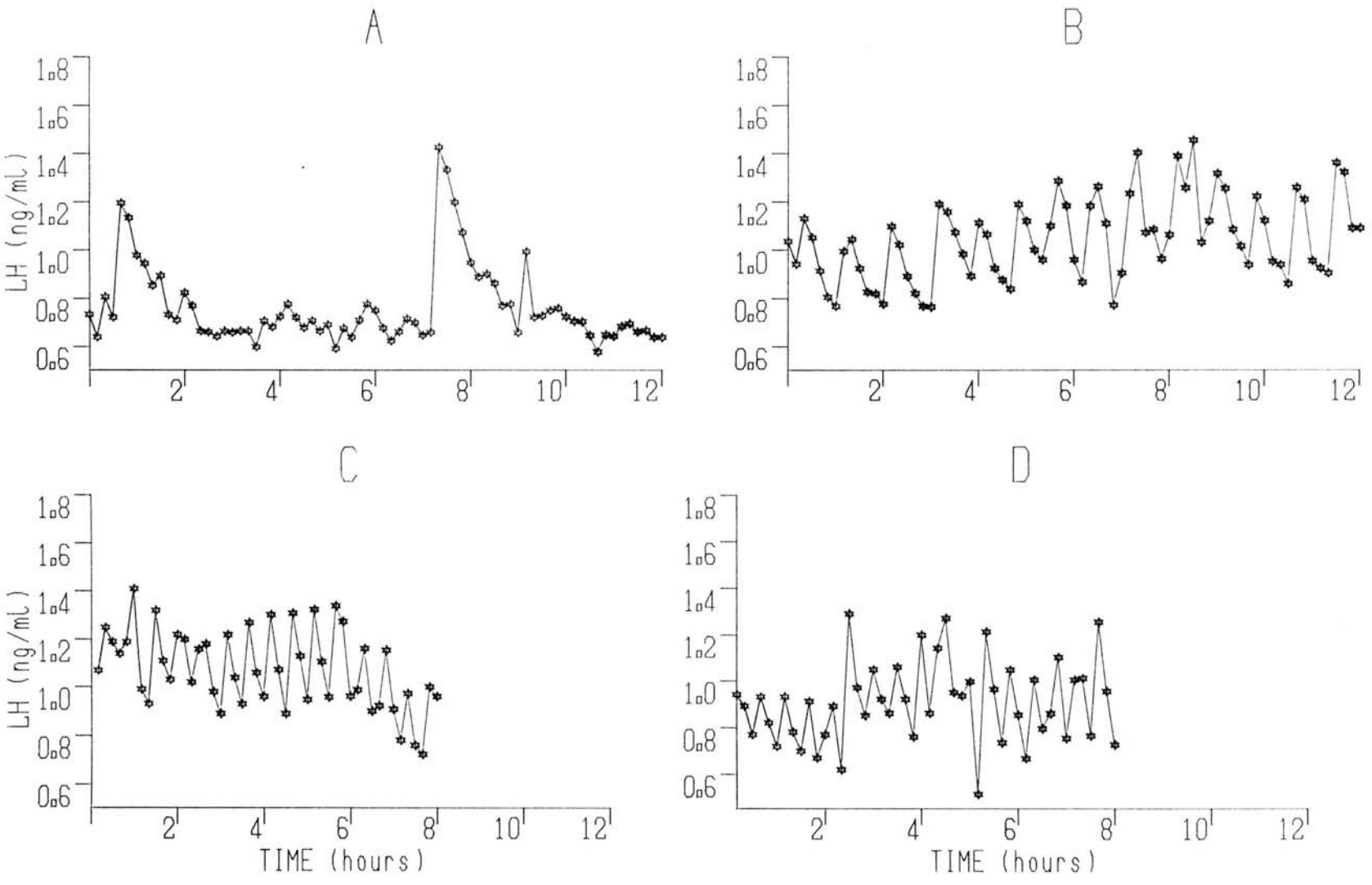

A
B
C
D
LH (ng/mL)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
2
4
6
8
10
12
TIME (hours)

TABLE 3.4

Serum progesterone concentrations at the time of sampling for LH and FSH profiles in the two anoestrous, immunised heifers (590 and 601), the cycling, immunised heifer (558) and the control heifer (603) shown in Figure 3.5.

	Progesterone concentration (ng/ml)		
Heifer	Luteal phase	Follicular phase	Anoestrous
601			2.3
590			0.3
558	16.0	5.4	
603	3.4	0.4	

ovariectomised heifers (Table 3.3). Interestingly, the mean LH concentrations of the anoestrous heifers were significantly lower than those of the ovariectomised heifers ($P<0.05$). Since time series analysis does not identify individual pulses, the pulse amplitudes could not be analysed, and are thus not included in Table 3.3.

The mean FSH concentrations of the anoestrous, immunised heifers were significantly different between cows, and significantly lower than those of the control heifers (Table 3.3). The immunised, cycling heifer displayed a significantly lower mean FSH concentration in the follicular than in the luteal phase ($P<0.05$); there was no such difference overall between the control heifers, although the difference between phases within cows ($P<0.05$) was different between cows ($P<0.05$).

3.3.5 Anoestrous Immunised Heifers

The four remaining anoestrous heifers were allowed to remain undisturbed to establish if falling antibody titres would lead to the resumption of oestrous cycles and an increased ovulation rate, as seen with heifers 558, 586, and 589. However, ovulation was not observed by 40 weeks after the second booster injection (for antibody titre, see Table 3.1) and the ovaries still possessed cystic structures, so it was decided to stimulate ovarian activity using a hormonal regime designed for the treatment of dairy cows with polycystic ovaries (Kesler, Garverick, Caudle, Bierschwal, Elmore and Youngquist, 1978). Each heifer was given a bolus intramuscular injection of a synthetic GnRH (Fertagyl; Intervet,

Cambridge) followed nine days later by a luteolytic dose of PGF2α (2ml Estrumate; ICI). Animals were blood sampled at 0, 1, 2, 3 and 6h after GnRH for LH surge detection, and three times weekly thereafter for four weeks for progesterone determinations. Observations for oestrus were continued for two months after the GnRH treatment.

All heifers responded to the GnRH by releasing a surge of LH which peaked at 23.7 ± 2.4ng/ml after 2 hours and fell to pretreatment concentrations within 6 hours. The progesterone data for these animals are shown in Figure 3.6. The rapid decline in progesterone seen in some heifers as a result of the prostaglandin treatment confirms that the cystic structures observed were secreting progesterone. Three of the four anoestrous heifers showed behavioural oestrus in response to the treatment, only one of which ovulated and showed regular oestrus thereafter (heifer 566). The heifer which did not exhibit oestrus (heifer 590) still possessed cystic ovaries, but displayed oestrus on two subsequent occasions at an interval of 21 days. The latter oestrus was observed to be anovulatory.

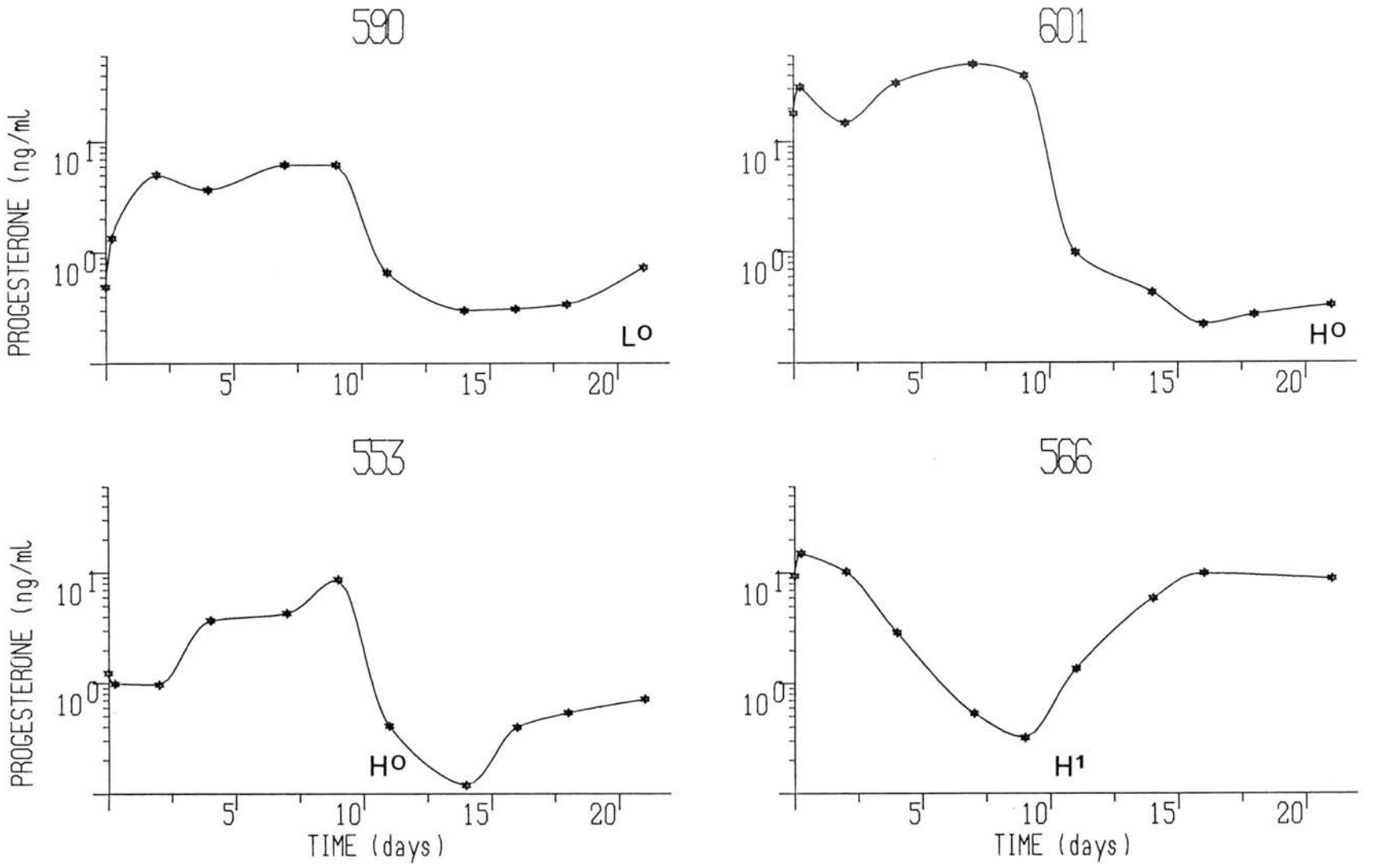
3.4 DISCUSSION

This study shows that active immunisation of heifers against a testosterone conjugate can have profound effects on the reproductive system. All immunised heifers that had a detectable immune response became anoestrous for at least six months following immunisation. Blocked or delayed oestrus has also been noted in rats (Hillier *et al.*, 1975) and sheep (Scaramuzzi, 1979) actively immunised against

FIGURE 3.6

Serum progesterone profiles from the four anoestrous, immunised heifers treated with GnRH and PGF2α. The GnRH was given at time 0, and the PGF2α given 9 days later.

H and L indicate the occurrence of oestrus, and laparoscopy in the absence of oestrus, respectively, with superscripts indicating the number of corpora lutea observed in the ovary at laparoscopy.


590
601
553
566
PROGESTERONE (ng/ml
10^1
10^0
5
10
15
20
L^o
H^o
H^o
H^1
TIME (days)
TIME (days)

testosterone. Those heifers which did resume oestrous activity, however, did so with a high incidence of multiple ovulations. Similar findings have been reported for testosterone immunised sheep (Scaramuzzi, 1979; Scaramuzzi et al., 1981), although preliminary results in the cow are conflicting (Sreenan et al., 1983; D'Occhio et al., 1986).

Anoestrus and the subsequent onset of oestrous activity and multiple ovulation may be related to antibody titre (Cox et al, 1982), but in the present study, despite similar antibody affinities, there were no clear differences in titre between multiple ovulating and anoestrous heifers (Figure 3.1). However, difficulties arise when antibody characteristics are correlated with biological effects. The specificity of an antiserum at a final dilution of 1:21000 in vitro may be quite different from that when undiluted in vivo.

Similarly, different antibody populations can become evident when Scatchard analyses are conducted on antisera at progressively lower dilutions (see for example, Webb et al., 1985b). In addition, neither of the above properties can be expected to remain constant following active immunisation. The antibody titres measured in the present study did not decline gradually, but fluctuated, as illustrated in Figure 3.1. Changes in specificity and/or affinity may explain why heifer 589 relapsed into anoestrus after exhibiting only one oestrus and ovulation.

Antibody titre per se may not fully account for the duration of anoestrus seen in the current study, as the antibody titre levels for most of the animals were significantly lower 40 weeks after immunisation than they were at 10 weeks (Table 3.1). Another factor may have been the presence of ovarian cysts, which can lead to long

term anoestrus and anovulation (see Seguin, 1980); GnRH and PGF2α administration caused the resumption of ovulation in at least one anoestrous heifer out of four, and caused consecutive displays of oestrus in another. Overstimulation of the ovaries by gonadotrophins has been implicated in the formation of cysts in cattle (Erb, Surve, Callahan, Randel & Garverick, 1971) and humans (Mahesh & Greenblatt, 1961). The gross anatomy of these cysts was compatible with a thin walled follicular cyst (Al-Dahash & David, 1977), but on dissection, at least one cyst proved to have a comparatively thick luteinised wall in addition to a large fluid volume (see Figure 3.3). The progesterone data also suggested the presence of a number of luteinised secretory structures.

The very high concentrations of progesterone seen in the immunised animals compared with those in the control animals reflect the total progesterone in the peripheral circulation, since the addition of hydrochloric acid to the extraction phase of the assay (see Chapter 2) causes the dissociation of progesterone from immunoglobulins. This does not, however, indicate how much of this progesterone is circulating in an active form and available for uptake into target cells.

The present findings of significantly increased mean gonadotrophin concentrations and LH pulse frequency in the anoestrous, immunised heifers compared with that of control heifers, would suggest an overall decrease in negative feedback to the hypothalamus/pituitary gland. It is known that in the cow LH and FSH are under the feedback control of oestradiol and progesterone (Beck et al., 1976; Beck and Convey, 1977; Ireland and Roche, 1982b; Butler et al., 1983), but not testosterone (Thompson et al., 1984); thus the testosterone antibodies in the present study could

be acting to decrease overall negative feedback by cross-reacting with and neutralising circulating oestradiol and progesterone in vivo. The elevated gonadotrophin secretion in the anoestrous, immunised heifers in the presence of high and low progesterone concentrations (Table 3.4) suggests at least partial neutralisation of circulating progesterone.

Complete removal of ovarian feedback upon the hypothalamus/pituitary gland, ovariectomy, is known to raise LH and FSH mean concentrations and LH pulse frequency in cattle (Hobson and Hansel, 1972a, b; Short et al., 1973; Forrest et al., 1980; Schallenberger and Peterson, 1982). Comparison of the ovariectomised heifers with the anoestrous, immunised heifers in the present study indicates that whilst the mean LH and FSH concentrations are significantly lower than those of an ovariectomised animal, the LH pulse frequency is no different than that of ovariectomised cows. The pulse interval of the follicular phase control heifer in the present study compares well with the estimates of Schallenberger et al. (1984), although, as in the case of the cycling immunised heifer, meaningful comparisons cannot be made between single figures.

The reason for this differential effect of the treatment on LH secretion is unclear. The amplitude of the LH pulses in the anoestrous heifers appears low when compared visually to that of the control heifers, although a comparison with the ovariectomised heifers is difficult to make without statistical analysis. A low pulse amplitude could be produced by exhaustion of pituitary LH content or by negative feedback of ovarian hormones (see Chapter One); neither of these can be ruled out in the present study. Similar effects on LH and FSH secretion have been reported in sheep

actively and passively immunised against testosterone (Martensz and Scaramuzzi, 1979; Pathiraja, 1982; Pathiraja et al., 1984).

Testosterone antibodies within the follicle may influence gonadotrophin secretion and ovarian activity by directly reducing ovarian output of oestradiol and/or inhibin. Testosterone can act in concert with FSH to stimulate granulosa cell aromatase activity as well as acting as a substrate (Daniel and Armstrong, 1980). Anti-testosterone serum has been shown to decrease ovarian oestradiol secretion when infused into the ovarian artery of sheep (Baird, 1977), and when added to cultures of combined granulosa and theca cells in rats (Liu & Hsueh, 1986). Inhibin can decrease both serum FSH and ovarian activity in sheep and cattle (Miller et al., 1979b; Ireland et al., 1983); the depressed FSH concentrations in this study may thus be a consequence of ovarian inhibin secretion.

In summary, this experiment has shown that active immunisation against testosterone caused prolonged periods of anoestrus in cattle. This anoestrus was associated with a high incidence of ovarian cysts and increased ovarian progesterone secretion. The mean LH pulse frequency of the immunised heifers was significantly higher than that of the control heifers, whilst mean FSH concentrations were significantly suppressed. This suppression of FSH secretion is a possible reason for the ovulatory failure, which suggests that the present immunisation regime against the androgens is not a suitable method of increasing ovulation rate in cattle. However, all heifers that resumed spontaneous ovarian cyclicity did so with an increased ovulation rate, thus the current treatment affected at least part of the control mechanism that limits fecundity in the cow.

CHAPTER FOUR

STEROID CONTROL OF GONADOTROPHIN SECRETION & OVARIAN FUNCTION

The results of Chapter Three extend those of Sreenan et al. (1983) and D'Occhio et al. (1983), which collectively show that active immunisation against testosterone produces highly variable results and is an inappropriate method for increasing prolificacy in the cow.

One possible component of the inconsistency in these results is the lack of control over antibody titre and specificity. However, the variation between animals within a treatment group was high even in heifers passively immunised against ovarian steroids (Webb et al., 1984). One interpretation of these results is that the hypothalamo-pituitary-ovarian axis is intolerant of changes in circulating hormones of the magnitude induced by active or passive immunisation. It is not known whether it is the hypothalamus/pituitary gland that 'over-reacts' to the immunisation treatment, thus disrupting ovarian function, or whether it is the ovary which 'over-reacts' to the treatment, either directly, or in response to immunisation-induced changes in gonadotrophin secretion.

The aim of this study was to investigate the hypothalamic/pituitary and ovarian response to various concentrations of circulating oestradiol. Oestradiol was chosen since it is the major feedback hormone of the ovary; testosterone, per se, is not believed to exert a major effect in the female (Butler et al., 1983). This experiment would also provide more information on the interaction of oestradiol and progesterone in the control of LH and FSH secretion in cattle.

4.2.1 Protocol

Twenty-three Hereford-Friesian yearling heifers were divided into three treatment groups of six, and a control group of five animals. The oestrous cycles of all animals were synchronised by two consecutive injections of PGF2α eleven days apart. On Day 9 of the synchronised oestrous cycle the control heifers received empty implants (Group A), and the treatment heifers received either a small (Group B), medium (Group C) or large (Group D) sized oestradiol implant. The implants were made to the specifications described in Chapter Two.

All heifers were allowed to complete the synchronised oestrous cycle, and were ovariectomised (see Chapter Two) during the luteal phase of the subsequent cycle. At ovariectomy, each heifer had a PRID inserted, and, 2 to 3 days later, was blood sampled every 10 min for 12h. All PRID's were removed between 5 and 7 days after ovariectomy, and the animals sampled again every 10 min, but for 6h only, on the fourth day after PRID removal. This protocol was designed to mimic the timing of events as they occur during the natural oestrous cycle.

Observations for oestrus were made 2 to 3 times daily throughout the experiment.

4.2.2 Blood Sampling

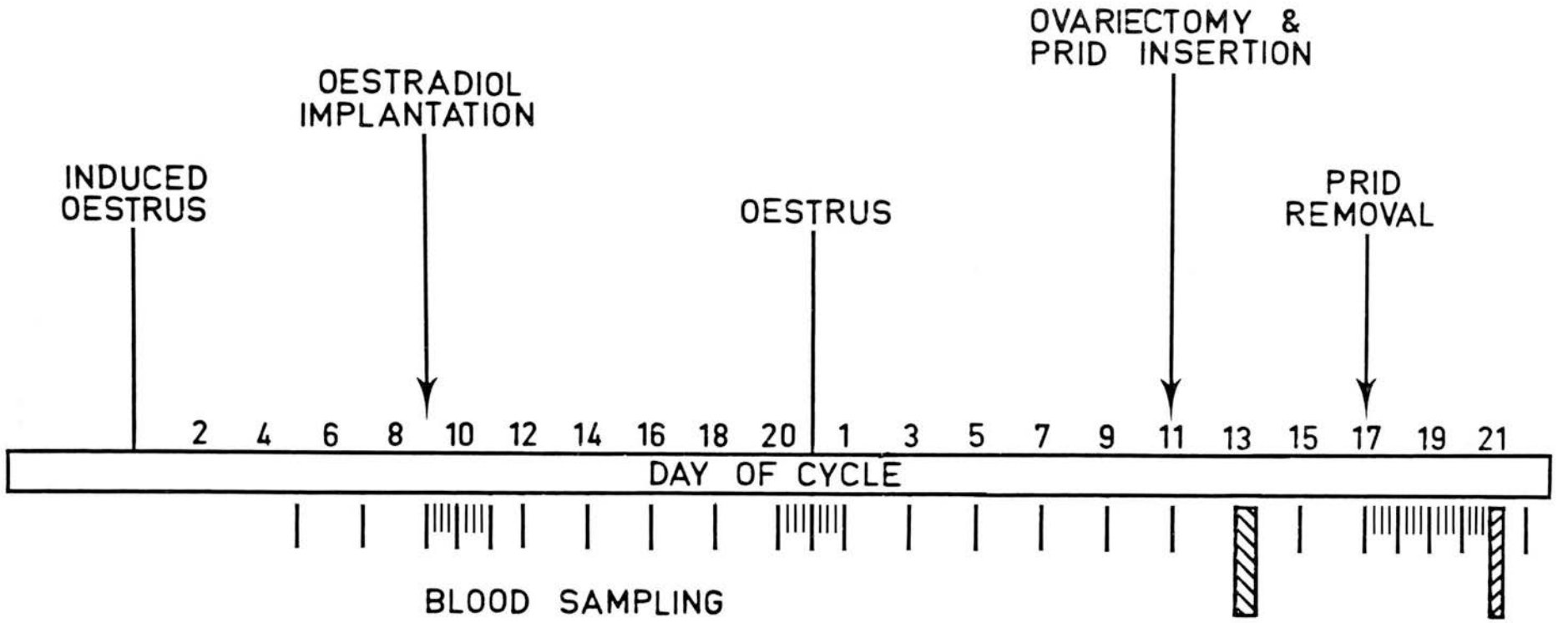
In addition to the intensive sampling, each animal was bled (50ml) three-times weekly for plasma progesterone and oestradiol determinations, starting four days prior to implant insertion and continuing until the day of the last intensive sampling period. To detect LH surges at implant insertion, oestrus, and after PRID removal, blood samples were taken every 6 hr for 3-5 days at each of these occasions. This protocol is illustrated in Figure 4.1.

4.2.3 Ovarian Studies

The ovaries removed from each animal were quickly transferred to the laboratory, and all follicles greater than 1mm in diameter were dissected free from connective tissue and counted. The ovaries and dissected follicles were kept immersed in Medium 199 (Flow Laboratories, Rickmansworth) at 37°C to maintain oestradiol secretion *in vitro* (Staigmiller *et al.*, 1982). The ten largest follicles from each heifer were incubated whole for 4h in 1ml Medium 199 individually at 37°C in an atmosphere of 95% air and 5% carbon dioxide. The media from all follicles were changed every hour, and stored frozen until assayed for oestradiol. The corpora lutea were removed from the ovaries and weighed.

FIGURE 4.1

Design of the experiment showing timing of oestradiol implantation, ovariectomy, PRID insertion and removal, and the blood sampling schedule. The blood samples were taken as 50ml samples for plasma progesterone and oestradiol assay every 2-3 days (▌), 10ml samples every 6 hr for the detection of the LH surge (▮), and as 10ml samples taken every 10 min for 12 hr (▒) or 6 hr (▒) through indwelling jugular cannulae for LH and FSH determinations.


OVARIECTOMY &
PRID INSERTION
OESTRADIOL
IMPLANTATION
INDUCED
OESTRUS
OESTRUS
PRID
REMOVAL
2 4 6 8 10 12 14 16 18 20 1 3 5 7 9 11 13 15 17 19 21
DAY OF CYCLE
BLOOD SAMPLING

4.2.4 Hormone Assays

The sensitivity of the LH assays was 0.04ng/tube, calculated over ten assays. The intra- (from 20 duplicate samples) and inter-assay coefficients of variation were 3.8% and 7.8%, respectively. All FSH determinations were completed in one assay, with a sensitivity of 0.6ng/tube, and intra-assay coefficient of variation of 8.6%.

The progesterone determinations were completed in three assays, with a mean extraction efficiency of 53%. The sensitivity of the assay was 0.14ng/ml and the intra-assay coefficient of variation (from 20 duplicate samples) of 4.7%. The inter-assay coefficient of variation for control samples containing 1.5 and 3ng/ml were 14% and 19%, respectively.

The oestradiol assays were conducted in two series. The plasma samples were extracted (see Chapter Two) with a mean efficiency of 70%, and completed in three assays. The mean sensitivity of the assays was 0.5pg/ml and the intra-assay (over 20 duplicate samples) coefficient of variation of 12.1%. The inter-assay coefficients of variation, for control samples containing 10 and 20pg/ml, were 15.1% and 12.5%, respectively. The media samples were assayed without extraction in four assays, with a sensitivity of 0.6pg/tube, and intra- (from 20 duplicate samples) and inter-assay (from samples containing 60pg/ml) coefficients of variation of 7%.

4.2.5 Statistics

All hormone data are presented as geometric means (±s.e.m.), and all pulse analyses were performed with the pulse programme described in Chapter Two. Differences within and between groups were examined by analysis of variance, with corrections for multiple comparisons.

4.3 RESULTS

The mean (±s.e.m.) Day of the oestrous cycle on which the heifers were ovariectomised was Day 11.5 ±0.8; one heifer was ovariectomised as early as Day 6, and another on Day 19 after having had a PRID inserted on Day 16. The mean Day of ovariectomy was not different between groups.

4.3.1 Plasma Steroid Concentrations

The circulating oestradiol and progesterone concentrations at various periods during the experiment are shown in Tables 4.1 and 4.2, respectively.

There were no significant differences in oestradiol concentrations between cows or groups before the implants were inserted. After implant insertion, the heifers of each treatment group showed significantly higher oestradiol concentrations than those of the control heifers ($P<0.05$) and there were significant differences between all groups except during the follicular phase of the cycle, when there were no differences between Groups A and B or

between Groups C and D, although Groups C and D were significantly different from Group A ($P<0.05$). Ovariectomy significantly lowered mean oestradiol concentrations in all groups ($P<0.05$). Differences between cows within groups were significant during the follicular phase, during the luteal phase before ovariectomy, and after the removal of the PRIDs.

There were no significant differences in progesterone concentrations between cows or groups during the induced luteal phase, either before or after implant insertion, nor during the subsequent follicular phase. There were no significant differences between Groups A, B or C during the luteal phase before ovariectomy, but the heifers of Group D had significantly lower mean progesterone concentrations than those of Group A heifers ($P<0.05$). There were also differences between cows within groups during this luteal phase. The PRID's produced progesterone concentrations which were not different between groups, but were different between cows within groups, and were not different from those of the previous luteal phase, except for Group C (Table 4.2). After PRID removal, progesterone concentrations were at the limit of detection of the assay.

4.3.2 Ovarian Activity

There was no effect of the oestradiol implants on the total number of ovarian follicles greater than 1mm in diameter, but there was a significant change in the distribution of follicle sizes; the two larger implants reduced the number of follicles greater than 6mm in diameter (Table 4.3 & Figure 4.2). The mean diameter of

TABLE 4.1

Geometric mean (±s.e.m.) oestradiol concentrations (pg/ml) in heifers receiving empty sachets (Group A), or small (Group B), medium (Group C) or large (Group D) sized sachets filled with crystalline oestradiol. The number of samples assayed for each group are given in parentheses.

Period in experiment	GROUP A	GROUP B	GROUP C	GROUP D
Luteal, before implant	0.97+0.23	0.85+0.20	0.79+0.18	1.01+0.22
(n)	(16)	(17)	(18)	(18)
Luteal, after implant	a 0.69+0.09	b 4.18+0.54	c 8.65+1.13	d 17.70+2.37
(n)	(23)	(24)	(23)	(22)
Follicular phase	a 5.52+1.17	ac 6.92+1.25	bc 11.40+1.96	b 16.87+2.29
(n)	(8)	(11)	(12)	(19)
Luteal, before ovariectomy	a 0.67+0.09	b 2.93+0.40	c 7.43+1.28	d 18.79+3.57
(n)	(28)	(26)	(17)	(14)
Ovariectomised, with PRID	*a 0.50	b 1.64+0.21	c 5.65+0.66	d 11.14+1.23
(n)	(5)	(14)	(17)	(19)
Ovariectomised, without PRID	*a 0.50	b 1.49+0.26	c 4.41+0.68	d 10.50+1.75
(n)	(4)	(12)	(15)	(13)

* Values at the limit of detection of the assay.

a,b,c,d
Means with different superscripts across a row differ ($P<0.05$).

TABLE 4.2

Geometric mean (±s.e.m.) progesterone concentrations (ng/ml) in heifers receiving empty sachets (Group A), or small (Group B), medium (Group C) or large (Group D) sized sachets filled with crystalline oestradiol. The numbers of samples assayed for each group are given in parentheses.

Period in experiment	GROUP A	B	C	D
Luteal, before implant (n)	2.99±1.13 (6)	2.69±0.80 (12)	3.64±1.14 (11)	2.41±0.80 (10)
Luteal, after implant (n)	3.32±1.30 (11)	3.61±1.01 (20)	3.10±0.98 (16)	1.44±0.47 (15)
Follicular phase (n)	* 0.14 (6)	0.21±0.05 (13)	* 0.14 (14)	0.27±0.06 (15)
Luteal, before ovariectomy (n)	a 1.38±0.98 (10)	a 2.39±1.05 (21)	ab 0.61±0.46 (9)	b 0.19±0.11 (13)
Ovariectomised, with PRID (n)	1.82±1.74 (4)	2.04±0.88 (13)	1.15±0.58 (10)	1.29±0.53 (14)
Ovariectomised, without PRID (n)	* 0.14 (5)	* 0.14 (12)	* 0.14 (12)	* 0.14 (11)

* Values at the limit of detection of the assay.

a,b Means with different superscripts across a row differ ($P<0.05$).

TABLE 4.3

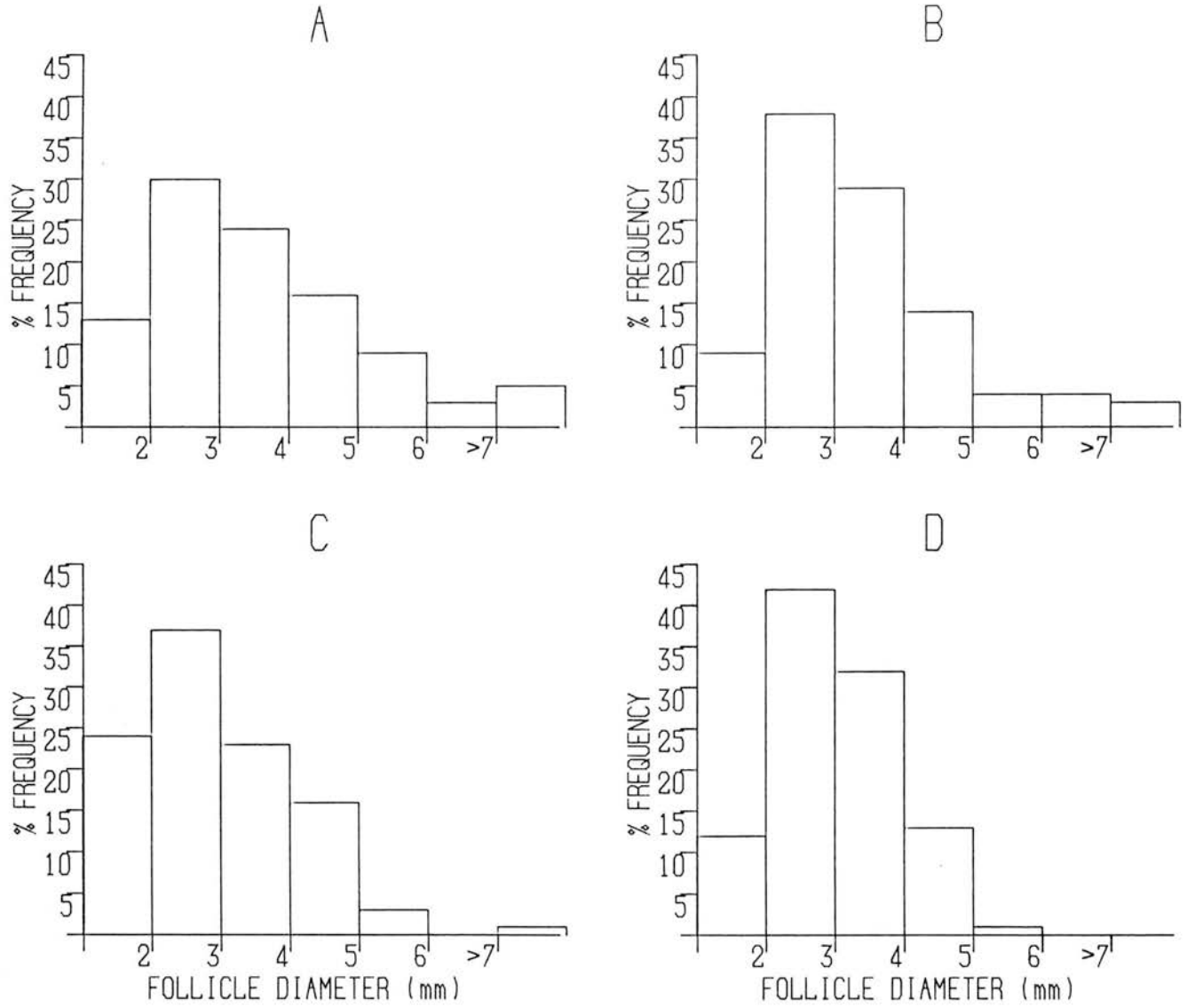
The ovulation rate, distribution of antral follicles and luteal weights of heifers receiving empty sachets (Group A), or small (Group B), medium (Group C) or large (Group D) sized sachets filled with crystalline oestradiol.

Character	GROUP			
	A	B	C	D
Number of heifers	5	6	6	6
Number ovulating	5	6	4	3
Ovulation rate	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	0.8 ± 0.2^{ab}	0.5 ± 0.2^{b}
Luteal weight (g/CL)	4.3 ± 0.3^{a}	3.2 ± 0.3^{b}	2.7 ± 0.6^{bc}	2.3 ± 0.8^{bc}
Total number of follicles/cow	35.8 ± 7.3^{a}	40.0 ± 5.9^{a}	53.5 ± 6.9^{a}	49.8 ± 12.6^{a}
Follicles/cow <6mm diameter	31.8 ± 6.3^{a}	36.7 ± 5.0^{ab}	51.7 ± 5.8^{b}	49.3 ± 11.6^{ab}
Follicles/cow, >6mm diameter	4.0 ± 0.7^{a}	3.3 ± 0.8^{ab}	1.8 ± 0.7^{bc}	0.5 ± 0.2^{c}
Diameter (mm) of follicle >8mm (n)	10.6+0.6 (5)	10.4+1.0 (5)	13.0+4.5 (2)	19.0+0.0 (1)

a,b,c
Means with different superscripts across a row differ ($P<0.05$).

FIGURE 4.2

The effect of empty implants (A), or small (B), medium (C) and large (D) sized oestradiol implants on the distribution of the antral follicle population in heifers. Results are the number of follicles of each size range expressed as a percentage of the total number of follicles greater than 1mm in diameter per group.


A
B
C
D
% FREQUENCY
45
40
35
30
25
20
15
10
5
2
3
4
5
6
>7
FOLLICLE DIAMETER (mm)

follicles greater than 8mm was significantly higher in Groups C and D, but this was confounded by the numbers of such follicles in each group; the only two large follicles in Group C had diameters of 8.5 and 17.5mm, and the single large follicle occurring in Group D had a diameter of 19mm. As indicated in Figure 4.3, these large follicles were secreting only small quantities of oestradiol, and could thus be considered atretic (Mertz et al., 1981; Tsonis et al., 1984). Further, there was a significant relationship between diameter and oestradiol production for the follicles of the control heifers ($r=0.44$; $P<0.05$), but not between those of the oestradiol implanted heifers.

Ovulation rate was significantly reduced in Group D compared to that of Group A, whilst the ovulation rate of Group C includes one heifer with two corpora lutea. The corpora lutea of all implanted heifers weighed significantly less than those of the control heifers ($P<0.05$; Table 4.3). Representative ovaries from each group are shown in Figure 4.4.

4.3.3 Gonadotrophin Secretion

Surges of LH were not detected after the insertion of the oestradiol implants, and those that were identified after PRID withdrawal were smaller than those which occur before ovulation; the maximum peak (8.2ng/ml) was recorded in two heifers, one from Group A and the other from Group C.

The results of the intensive bleeding periods are given in Tables 4.4 and 4.5, for heifers with and without PRID's, respectively. These data are illustrated in Figures 4.5 and 4.6.

FIGURE 4.3

The effect of empty implants (A), or small (B), medium (C) and large (D) sized oestradiol implants on the oestradiol secretion of the ten largest follicles per heifer taken at ovariectomy. Results are expressed as the percentage of all cultured follicles, within a group, that secreted the given quantities of oestradiol (pg/ml/hr).

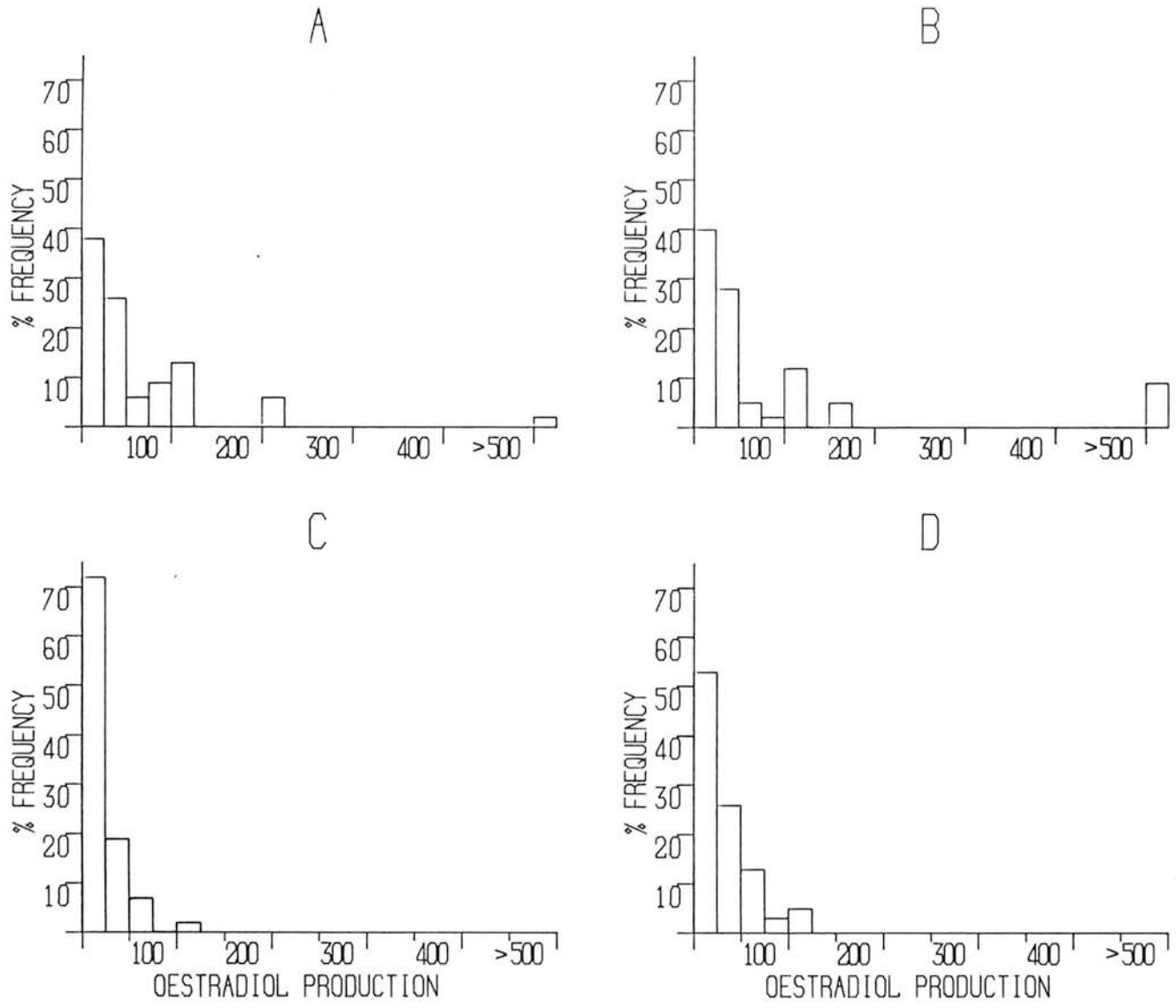
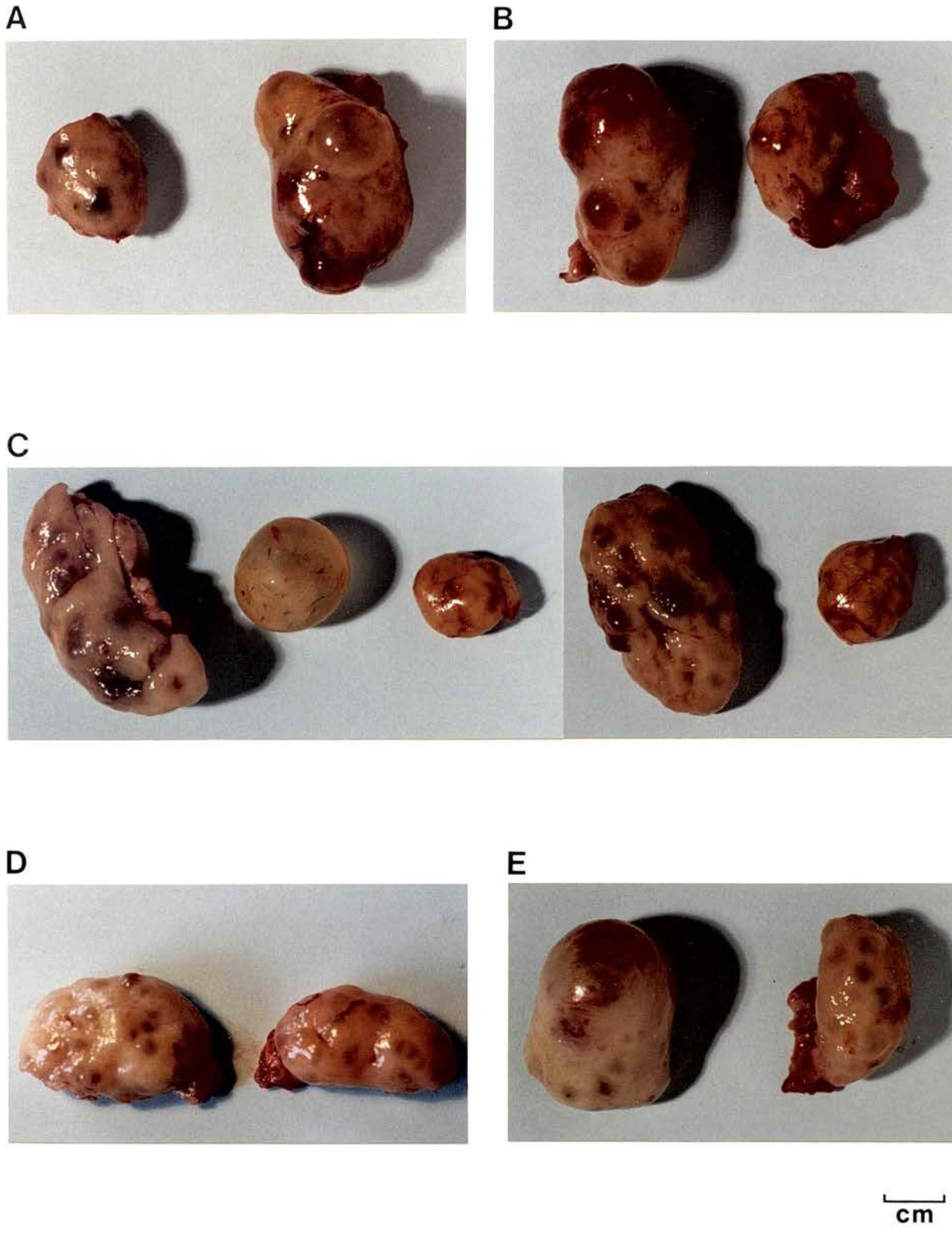

A
B
C
D
% FREQUENCY
70
60
50
40
30
20
10
100
200
300
400
>500
OESTRADIOL PRODUCTION

FIGURE 4.4

Ovaries from heifers of each group showing various features of the treatments. Ovaries from a control heifer (A) show a large follicle and single corpus luteum common to all control heifers. The corpora lutea of the heifers receiving the small implant (B) weighed significantly less than those of the control heifers. This effect was more marked in the ovaries of heifers with medium sized implants (C); this heifer had two corpora lutea and an atretic follicle of diameter 17.5mm. Ovaries from two heifers given large implants show the lack of corpora lutea and large follicles (D) occurring in all animals except one heifer which possessed an atretic follicle of 19mm diameter (E).


A
B
C
D
E
cm

In the presence of progesterone, the heifers of all oestradiol treated groups showed significantly depressed mean LH concentrations, and LH pulse frequency and amplitude, than those of the control heifers ($P<0.05$). In the absence of progesterone, LH pulse frequency was not decreased by the oestradiol implants ($P>0.05$).

Progesterone alone significantly decreased mean LH secretion in all heifers, and decreased LH pulse frequency in all heifers except in those of Group D. LH pulse amplitude was not significantly decreased by progesterone in the absence of oestradiol (Group A), nor in the heifers receiving the largest oestradiol implant (Group D).

Progesterone reduced mean FSH concentrations in all heifers, although this reached significance only in the heifers of Group D. Oestradiol decreased mean FSH concentrations in the presence or absence of progesterone, although this suppression was significant only between Groups A and D.

4.4 DISCUSSION

These results demonstrate the significant effect of exogenous oestradiol and progesterone upon reproductive function in the cow, and show the manner in which gonadotrophin secretion is controlled by gonadal steroids.

The implants in this experiment produced physiological concentrations of oestradiol in the ovariectomised heifers, which were comparable with those of intact heifers during the luteal phase, follicular phase and during oestrus (see section 1.1.1) for

TABLE 4.4

Secretory patterns of LH and FSH in ovariectomised heifers, with PRIDs, implanted with different sized oestradiol implants. Data are expressed as geometric means (±s.e.m.).

		LH			FSH
Group	No. of heifers	Mean (ng/ml)	Pulse frequency (per 6h)	Pulse amplitude (ng/ml)	Mean (ng/ml)
A	5	0.87±0.02 [a]	4.00±0.45 [f]	0.64±0.05 [a]	41.0±8.2 [d]
B	6	0.49±0.01 [b]	2.40±0.81 [e]	0.33±0.03 [b]	30.1±4.2 [de]
C	6	0.48±0.01 [b]	0.85±0.40 [d]	0.21±0.03 [c]	29.5±4.1 [de]
D	6	0.62±0.01 [c]	1.65±0.83 [e]	0.28±0.04 [b]	21.3±3.0 [e]

a,b,c,d,e,f Mean values down a column without the same superscript differ ($P<0.05$)

TABLE 4.5

Secretory patterns of LH and FSH in ovariectomised heifers, without PRIDs, implanted with different sized oestradiol implants. Data are expressed as geometric means±s.e.m.

		LH			FSH
Group	No. of heifers	Mean (ng/ml)	Pulse frequency (per 6h)	Pulse amplitude (ng/ml)	Mean (ng/ml)
A	5	1.39±0.04^{x}	6.40±0.24	0.81±0.05^{x}	72.1±18.4^{x}
B	6	1.02±0.03^{y}	5.30±0.67	0.78±0.07^{x}	51.8±9.2^{x}
C	6	0.82±0.02^{z}	5.30±0.56	0.37±0.04^{y}	56.2±9.9^{x}
D	6	0.86±0.01^{z}	3.80±1.10	0.32±0.03^{y}	28.7±5.1^{y}

x,y,z Mean values down a column without the same superscript differ ($p<0.05$)

FIGURE 4.5

The effects of different plasma concentrations of oestradiol on mean LH (A) and FSH (B) concentrations in ovariectomised heifers with (————) and without (------) a PRID. The data are expressed as geometric means (±s.e.m.). Asterisks indicate that mean gonadotrophin concentrations in the presence of progesterone were significantly lower than those in the absence of progesterone.

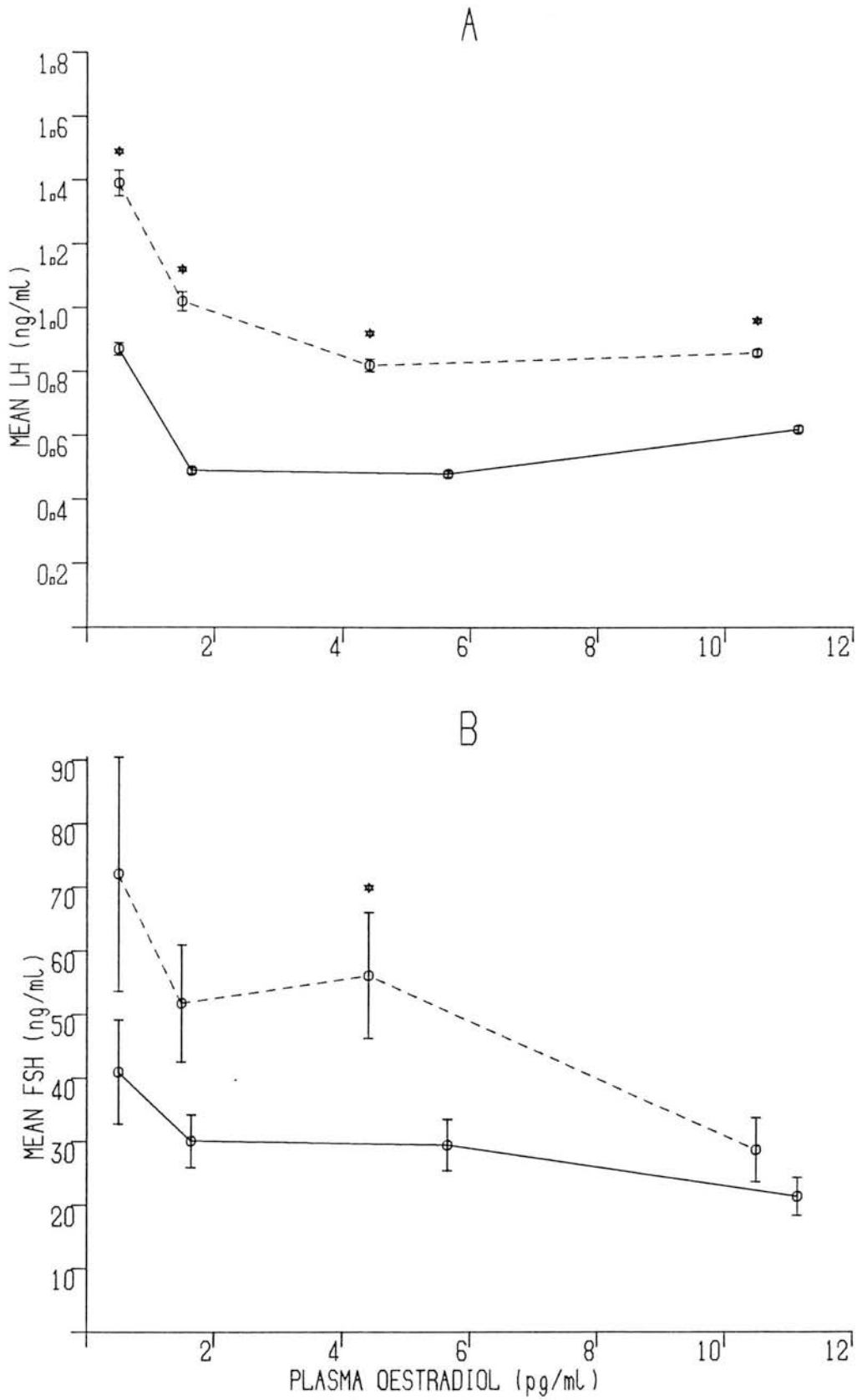
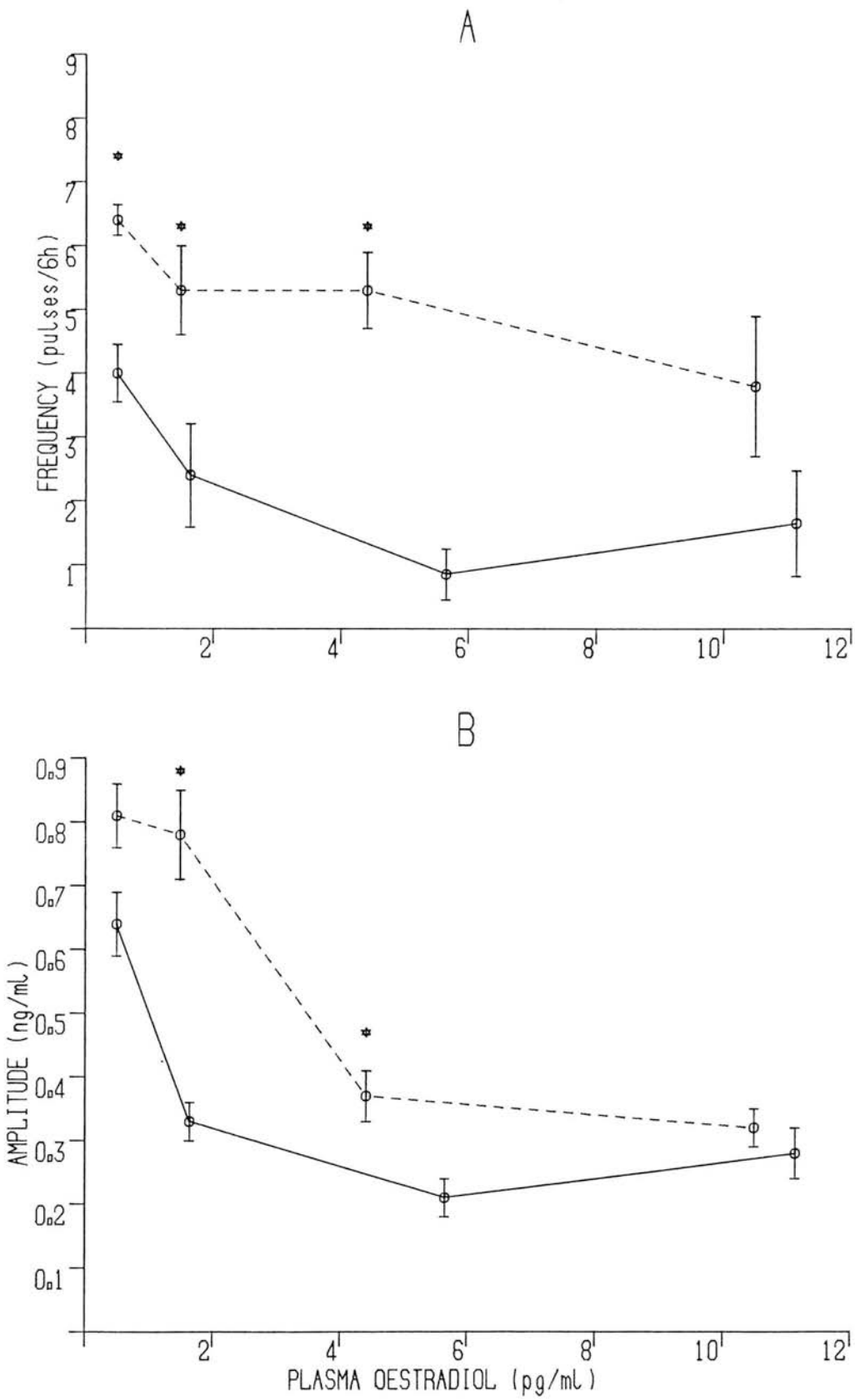

A
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
MEAN LH (ng/mL)
2
4
6
8
10
12
B
90
80
70
60
50
40
30
20
10
MEAN FSH (ng/mL)
2
4
6
8
10
12
PLASMA OESTRADIOL (pg/mL)

FIGURE 4.6

The effect of different plasma concentrations of oestradiol on LH pulse frequency (A) and amplitude (B) in ovariectomised heifers with (————) and without (------) a PRID. The data are expressed as geometric means (±s.e.m.). Asterisks indicate that the pulse characteristics in the presence of progesterone were significantly lower than those in the absence of progesterone.


A
FREQUENCY (pulses/6h)
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
B
AMPLITUDE (ng/mL)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
2
4
6
8
10
12
PLASMA OESTRADIOL (pg/mL)

Groups B, C and D, respectively. The oestradiol concentrations were higher and more variable in each group prior to ovariectomy, owing to endogenous oestradiol secretion. The PRIDs produced progesterone concentrations which were lower than those reported by Roche & Ireland (1981a) and those measured during the induced luteal phase in the current study, although this difference reached significance only in Group C.

Both oestradiol and progesterone decreased mean LH concentrations, and in combination the effect was additive. This extends the previous results in the cow (Beck et al., 1976), and confirms the relationship between progesterone and LH suspected by Roche & Ireland (1981b). In the sheep, some authors reported no effect of progesterone on mean LH concentrations (Wheaton & Mullett, 1982; Rawlings et al., 1984), whereas others showed a significant suppression (Karsch, Legan, Hauger & Foster, 1977). The effect of a combination of steroids in sheep was reported to be interactive (Goodman et al., 1980; Goodman et al., 1981b).

A closer examination of the pattern of LH secretion induced by the steroid treatment revealed that progesterone alone could not significantly depress pulse amplitude, but did suppress pulse frequency, and significantly enhanced the suppressive action of oestradiol on pulse amplitude. Oestradiol alone decreased pulse amplitude, but had no significant effect on pulse frequency even at the highest dose level. A negative action of progesterone on LH pulse frequency, but not on amplitude, has been reported in intact cattle (Ireland & Roche, 1982b). These results present a major advance in our understanding of pulsatile LH release in cattle. The only similar report in the literature (Imakawa et al., 1986) suggested that oestradiol alone decreased pulse frequency and

increased pulse amplitude in cattle, but these animals had been implanted with steroid and ovariectomised for thirty-one days before being sampled; it has been shown in sheep that pituitary responsiveness to steroids after ovariectomy changes with time (Karsch et al., 1977).

The results of the present experiment are in general agreement with those described in sheep, but provides only limited support for the hypothesis advanced by Goodman & Karsch (1980), who proposed that progesterone acts in the hypothalamus to decrease the frequency of GnRH pulses, and that oestradiol acts on the pituitary gland to modify the response of the cells to GnRH (see also section 1.1.3.3). This hypothesis only holds in the cow if each steroid is considered alone; oestradiol could suppress LH pulse frequency in the presence of progesterone, and could only have done so by altering GnRH pulse frequency (see section 1.1.2.2). Thus progesterone appears to be necessary for the suppressive action of oestradiol on LH pulse frequency, and enhances the oestradiol-induced decrease in LH pulse amplitude.

The significant depression of mean LH concentrations by each steroid alone suggests a mode of release other than that by pulses per se. This basal LH release contributes to the immunoreactive LH measured between pulses, and supports the concept of several pituitary pools of LH (see section 1.1.2.2). The role of basal LH secretion is unclear, but may be necessary to maintain ovarian receptors for LH, in the same manner that basal GnRH secretion is believed to be necessary for the maintenance of pituitary GnRH receptors (Clayton, 1982; Clarke et al., 1984a; Popkin & Fraser, 1985).

There was no clear exhibition of the positive feedback effects

of oestradiol in this experiment, although the removal of progesterone caused an increase in LH pulse frequency. It is possible that the sampling regime was insufficient to detect and characterise LH surges, or that, as the circulating oestradiol concentrations were static and not rising as in the follicular phase of the oestrous cycle, the positive feedback stimulus to the hypothalamus/pituitary gland was inappropriate for the induction of a LH surge.

The information in the literature on the steroid control of FSH secretion is conflicting. The current finding of no significant effect of progesterone alone, and a significant decrease by oestradiol alone, is in general agreement with published results in the sheep (Fraser et al., 1981; Goodman et al., 1981b; Moss et al., 1981) and cow (Ireland and Roche, 1982b; Butler et al., 1983; Schoenemann et al., 1985).

In sheep, a combination of oestradiol and progesterone was more effective than oestradiol alone in suppressing mean FSH concentrations (Moss et al., 1981; Rawlings et al., 1984), although not to levels observed in the oestrous cycle (Goodman et al., 1981b). This provided strong support for the role of inhibin as a feedback hormone in the ewe (see sections 1.1.3.3.2 and 1.1.4.3). In contrast to the work in the sheep, the present data do not reveal a clear interaction between oestradiol and progesterone. Further, both steroids together, or follicular-phase concentrations of oestradiol alone, reduced mean FSH concentrations to within the range observed during the oestrous cycle (Walters et al., 1984; Schallenberger et al., 1984). These data question the role of inhibin as a major feedback hormone in the cow, in contrast to the sheep.

The mean total number of antral follicles in the ovaries were comparable with that described by McNatty et al. (1984b), who also found that this was not significantly altered by the stage of the oestrous cycle. The present results extend this by showing that the total number of antral follicles was not affected by the oestradiol treatment. However, the treatment did change the distribution of antral follicles and the ovulation rate, in a manner similar to that observed in sheep (Webb & Gauld, 1985b, 1987). In these sheep studies, there was no significant effect of the oestradiol on FSH concentrations, although LH concentrations were not reported. In the present experiment, changes in ovarian characters were obtained without a significant effect on FSH concentrations in the two lower implant sizes. However, the ovarian data were not collected at the same time as the endocrine data, so strict parallels cannot be drawn. Further, the relationship observed in the control heifers between follicular diameter and oestradiol production in vitro was not seen in any of the implanted heifers. The absence of such a relationship was reported to be one characteristic of atretic follicles in the the cow (McNatty et al., 1984b; Henderson et al., 1987). Thus the present treatment may have increased the proportion of atretic follicles in the ovaries.

These changes in ovarian characters may also have been influenced by the steroid-induced changes in LH secretion, although preliminary data suggest that LH is not an absolute requirement for the development of large antral follicles from a pool of small antral follicles in sheep (Picton, Tsonis & McNeilly, 1986).

Increasing plasma concentrations of oestradiol significantly decreased the mean weight of the corpora lutea in all treated groups. It is known that LH is a major luteotrophic hormone in the

cow (see 1.1.2.1), and that a reduction of circulating LH causes significant decreases in plasma progesterone (Hoffmann et al., 1974). Since plasma progesterone concentrations before ovariectomy in the present experiment were lower only in the large implant group, it is possible that the decreased luteal weights in the other implanted heifers were not caused by decreased LH secretion. There is some preliminary evidence to suggest that oestradiol can directly inhibit the growth and proliferation of luteal cells in vitro (Luck, 1986).

In summary, physiological concentrations of oestradiol and progesterone in the cow were shown to decrease significantly all characteristics of gonadotrophin secretion studied, and, hence or otherwise, to reduce significantly ovulation rate, and the number of large and oestrogen-active ovarian follicles. Current immunisation techniques could thus alter steroid concentations beyond those required for regular ovarian function, and thus result in the type of response observed in Chapter Three.

CHAPTER FIVE

STUDIES ON THE BIOLOGY OF PARTIALLY PURIFIED FOLLICULAR FLUID

5.1 INTRODUCTION

The results described in the previous experiments, in addition to those available in the literature, strongly suggest that prolificacy cannot be successfully raised in cattle with current steroid immunisation techniques. Two further approaches are available; these involve, firstly, a detailed study of the ovarian response of cattle to passive immunisation against a wide range of anti-steroid titres, and, secondly, the immunisation of cattle against an inhibin-enriched fraction of follicular fluid. Considering recent work in sheep (see section 1.2.2.3), the latter approach could be the more productive.

This chapter describes studies which were designed to examine the biological activities of the partially purified follicular fluids (PPFF) used in Chapters Six and Seven, with respect to pituitary FSH secretion, follicular steroidogenesis, and ovulation rate.

5.2 MATERIALS AND METHODS

The sources of follicular fluids used in these studies, and the method of purification have been described in Chapter Two. The protein content of the raw follicular fluids and that of the partially purified fractions were examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970).

5.2.1 Pituitary Cell Assay

The sheep pituitary cell bioassay was performed by Dr. C.G.Tsonis (MRC Reproductive Biology Unit, Edinburgh) by the method of Tsonis *et al.* (1986a), using ovine rete testis fluid as the standard preparation.

5.2.2 Ovulation Rate Assay

The ovulation rate assay was based on that of Cummins (1983), with modifications. Mature outbred female mice (Bantin & Kingman Ltd., Hull) weighing 20-25g were taken at random and caged in groups of five. The administration of each test preparation was spread over four days to correct for variation in response between days of the mouse oestrous cycle. Each mouse received an intra-peritoneal injection of the test preparation in 0.1 ml sterile saline at 0900h on the first day of assay. Control mice received 0.1ml sterile saline. All mice were given 10 IU hCG (Chorulon; Intervet, Cambridge) in 0.1ml by intra-peritoneal injection at 1700h on the same day. On the morning of the second day of assay, each mouse was killed by cervical dislocation, and the oviducts removed. Ovulation rate was determined by counting the number of ova contained within the *cumulus oophorus*; this was facilitated by the use of hyaluronidase (Hyalase; Fisons) to disperse the cells of the *cumulus*. The ovulation data were transformed into logarithms before linear regression analysis.

5.2.3 Ovarian Studies

The follicles used in this study were obtained at ovariectomy from untreated ewes and heifers of mixed breeds and ages, during the luteal phase of the oestrous cycle. Follicles were dissected free of connective tissue, and those greater than 3mm in diameter were halved, washed in medium (M199; Flow Laboratories, Rickmansworth) and weighed. One half was incubated in medium alone, and the other half was incubated in the presence of either ovine-II or bovine PPFF (0.1mg/ml of medium). Follicles less than 3mm in diameter were ruptured and washed in medium; half of these follicles from each pair of ovaries were incubated in medium alone, and the remainder were incubated in medium with PPFF as above. All incubations were conducted for 4h at 37°C in an atmosphere of 95% air and 5% carbon dioxide. The follicles were placed into fresh medium every hour, and each medium sample was kept frozen until assayed for oestradiol. The numbers of follicles incubated under each condition are given in Table 5.1.

5.2.4 Hormone Assays

The media samples were assayed for oestradiol content, without extraction, in three assays with a mean sensitivity of 0.7pg/tube. The intra- (calculated from 20 duplicate pairs) and inter-assay (for samples containing 20pg/ml) coefficients of variation were 9.9% and 16.3%, respectively.

The bovine, porcine and ovine preparations were assayed for gonadotrophin content; the ovine LH and FSH assays were performed

TABLE 5.1

Design of the follicle culture experiment.

Follicle species	Inhibin species	Number of follicles in size range				
		<4mm	4.5-6mm	6.5-8mm	>8mm	Total
Bovine	Control	13	32	9	5	59
Bovine	Bovine	1	14	4	3	22
Bovine	Ovine	8	14	4	5	31
Ovine	Control	43	10	1	0	54
Ovine	Bovine	21	2	0	0	23
Ovine	Ovine	22	6	1	0	29

by Dr. J.R. McNeilly (AFRC, IAPGR, Roslin; LH: Webb et al., 1985a; FSH: McNeilly et al., 1976), and the porcine assays by Dr. G.R. Foxcroft (Faculty of Agricultural Science, University of Nottingham; Shaw & Foxcroft, 1985). The bovine PPFF was included in the assays described in Chapter Four.

5.3 RESULTS

The results of the SDS-PAGE are shown in Figure 5.1; there were at least quantitative differences in protein content between the preparations over a large molecular weight range. The LH and FSH concentrations of all the preparations tested were at the limit of detection of the respective assays.

5.3.1 Pituitary Cell Assay

The results of the sheep pituitary cell assay were expressed as Units/mg (Table 5.2). The two batches of ovine PPFF were not different from each other, but were significantly more potent ($P<0.05$) than the other preparations tested. The porcine and equine materials were equipotent, and were significantly less potent ($P<0.05$) than the bovine preparation.

FIGURE 5.1

Electrophoretic profiles of the ovine-I (O-I), ovine-II (O-II), porcine (P) and equine (E) follicular fluid preparations used in these studies. Molecular weight markers (Mr) are shown in kDa.

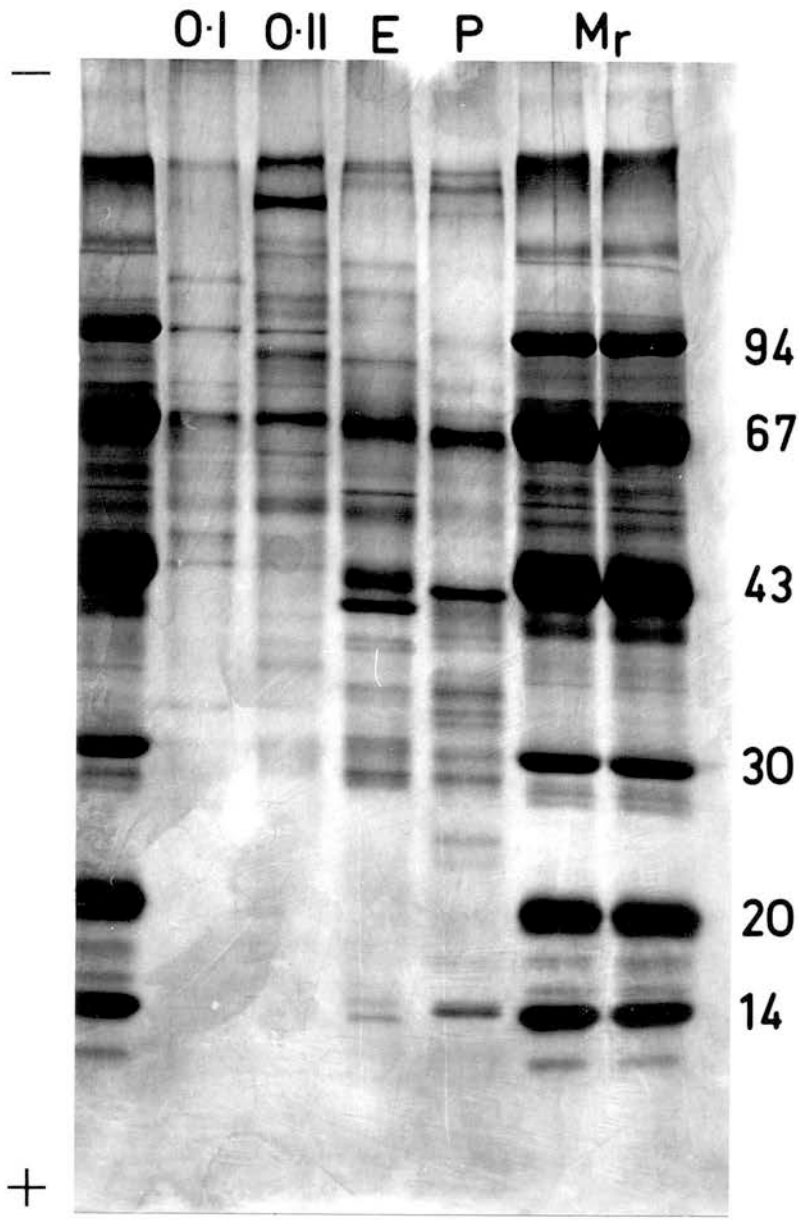

0·I
0·II
E
P
Mr
—
+
94
67
43
30
20
14

TABLE 5.2

Mean (±s.e.m.) potencies of the partially purified follicular fluids assayed in the sheep pituitary cell culture system of Tsonis et al. (1986a). The ovine-I preparation was used in Chapter 6, whilst the remainder were used in Chapter 7. The standard errors were calculated from the 95% confidence limits of the potency estimates (see section 2.3.6).

Preparation	Units/mg
Ovine-I	2845 ± 373^{a}
Ovine-II	2252 ± 271^{a}
Bovine	840 ± 105^{b}
Equine	34 ± 4.3^{c}
Porcine	37 ± 4.9^{c}

a,b,c Mean values with different superscripts differ ($p<0.05$)

5.3.2 Ovulation Rate Assay

For each preparation tested, the number of doses, the mean number of mice used at each dose level, and the mean regression coefficients, are presented in Table 5.3. The potencies were not different between the bovine, ovine-I, ovine-II and equine preparations, but were different for the porcine PPFF. The regression line for the porcine material, and a mean regression line for the remaining preparations, are given in Figure 5.2 over the dose ranges used.

5.3.3 Ovarian Studies

There was no effect on oestradiol secretion of incubating the follicles with PPFF, regardless of whether the follicles were classed on the basis of size, species, or preparation used. The follicles were also classified on the steroidogenic activity of the control incubations, the distribution of which is shown in Figure 5.3. Incubation with PPFF had no effect upon this distribution. There was no significant relationship between follicle diameter and oestradiol production *in vitro* for either the sheep or the cattle follicles. This, and the relatively low quantity of oestradiol secreted, suggest that the follicles studied were atretic (McNatty *et al.*, 1984b; Webb & Gauld, 1985a, b; Henderson *et al.*, 1987).

TABLE 5.3

Linear regression analysis of the effects of partially purified bovine, ovine-I, ovine-II, equine and porcine follicular fluids on the ovulation rate of mice. Data were transformed (log) before analysis and are expressed as mean (±s.e.m.) regression coefficients.

Preparation	Mean 'n' per dose	Number of doses	Regression	
			slope	intercept
Bovine	10	10	-4.7 ± 1.9^{a}	14.1 ± 3.6^{x}
Ovine-I	11	9	-5.1 ± 0.7^{a}	14.2 ± 1.3^{x}
Ovine-II	11	9	-2.1 ± 1.5^{a}	7.6 ± 2.8^{x}
Equine	10	4	-8.2 ± 4.4^{a}	20.1 ± 8.5^{x}
Porcine	10	5	5.6 ± 2.8^{b}	-3.9 ± 5.1^{y}

a,b,x,y Means down a column without the same superscript differ ($P<0.05$)

FIGURE 5.2

Pooled linear regression of dose of ovine-I, ovine-II, bovine and equine PPFF (————) and porcine PPFF (-------) upon ovulation rate in the mouse. The mean (±s.e.m.) ovulation rate of the control mice is also given (×).

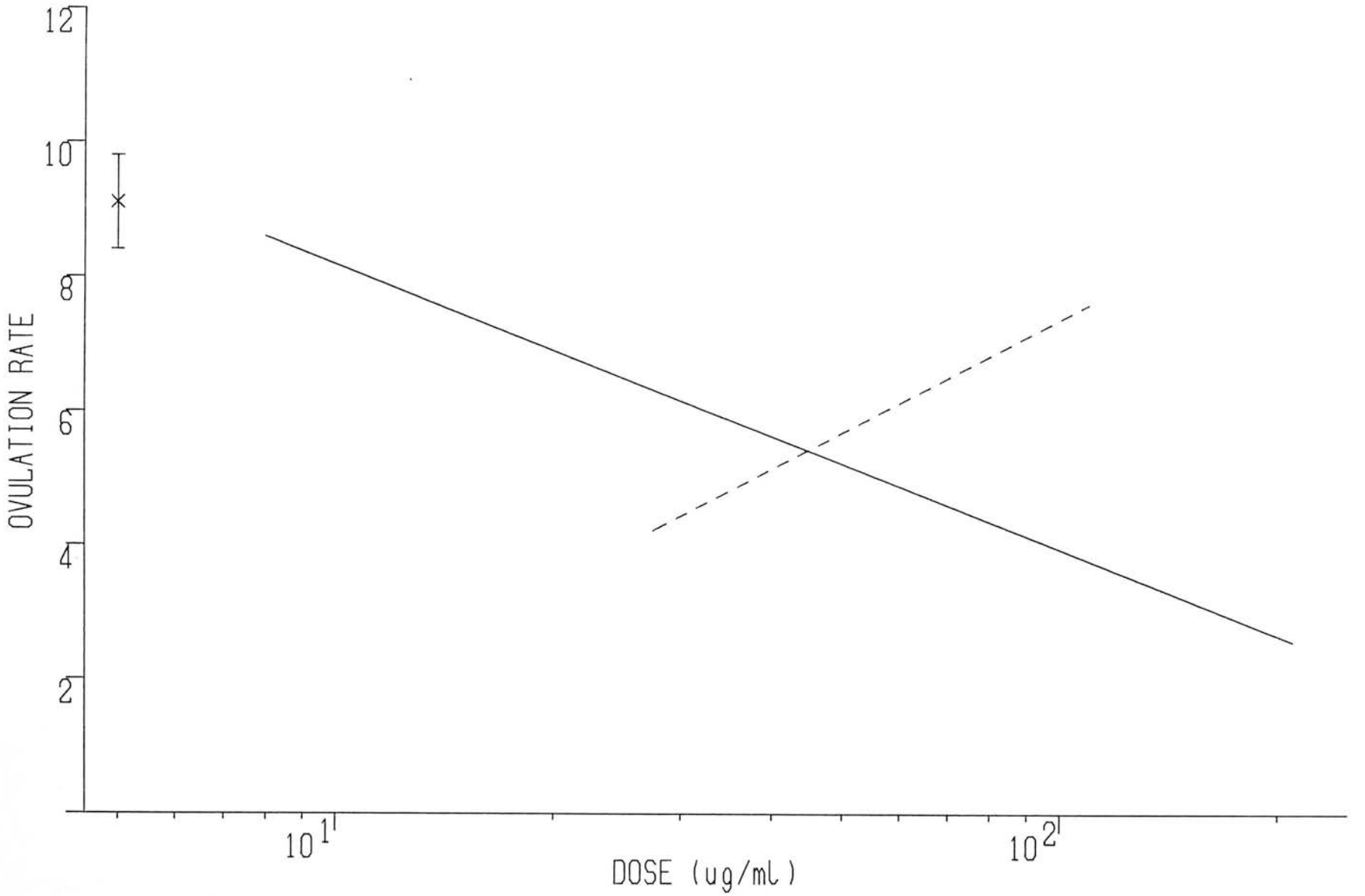
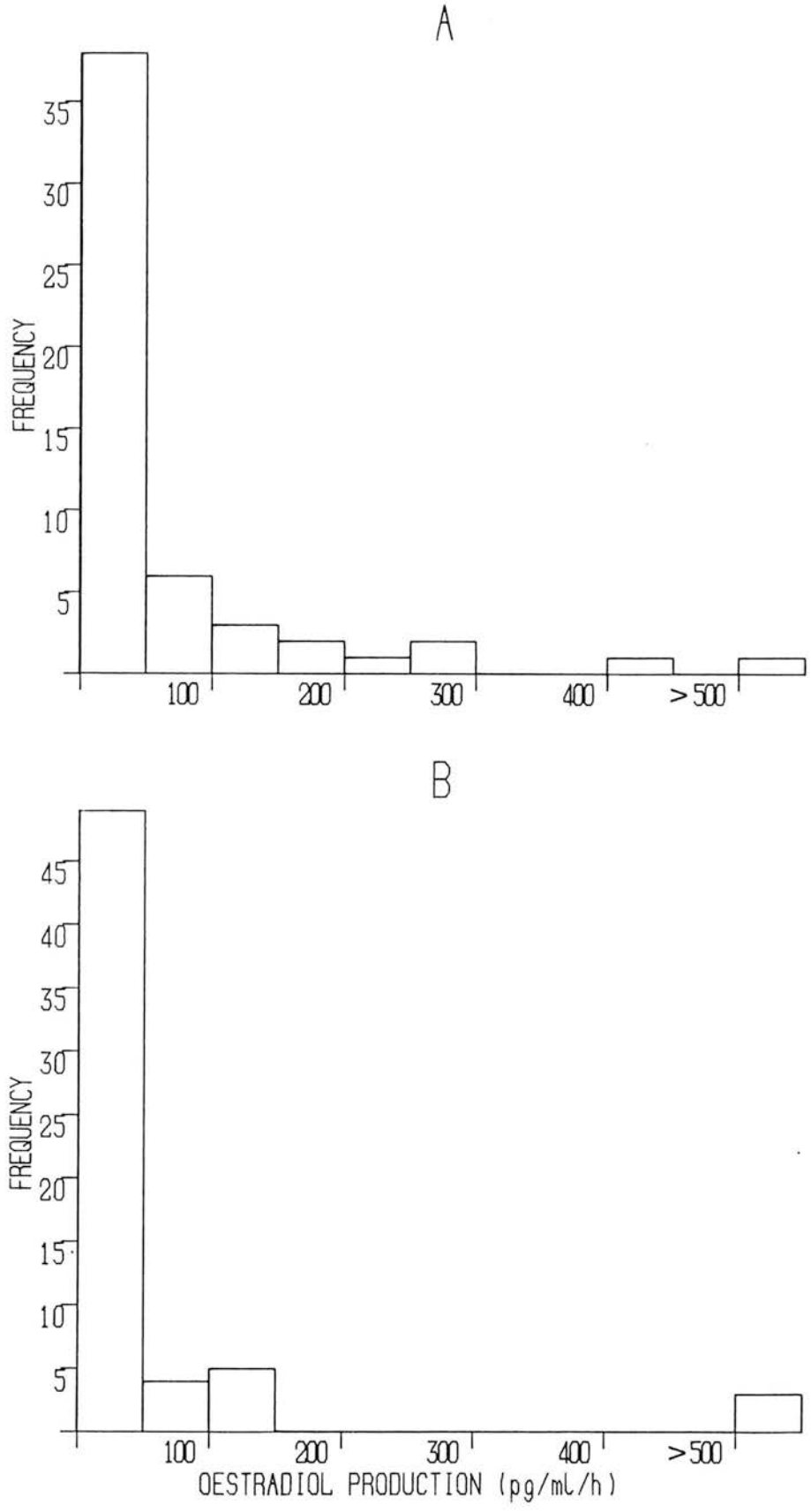

12
10
8
6
4
2
OVULATION RATE
10^1
10^2
DOSE (ug/mL)

FIGURE 5.3

Frequency histogram of oestradiol release from follicles of sheep (A) and cattle (B) cultured in Medium 199. Bovine or ovine-II PPFF had no effect on this distribution.


A
FREQUENCY
35
30
25
20
15
10
5
100
200
300
400
>500
B
FREQUENCY
45
40
35
30
25
20
15
10
5
100
200
300
400
>500
OESTRADIOL PRODUCTION (pg/mL/h)

5.4 DISCUSSION

The bioassay data indicate that the preparations used in these studies were capable of decreasing FSH secretion from sheep pituitary cells in culture, and of decreasing ovulation rate in the mouse. However, the two test systems give different relative potencies. The mouse bioassay suggests that there were no differences in potency between the ovine, bovine and equine preparations, and that the porcine material was significantly different from each of the above. The porcine PPFF displayed a positive regression slope, that is, ovulation rate increased as the dose of injected material increased. This is not compatible with inhibin-like activity as defined (see section 1.1.4). This porcine material may exhibit a quadratic dose-response curve, and that the doses employed in this study covered only one part of this curve. If this were the case, it can be inferred that the porcine preparation was more potent than any of the others tested.

The data from the pituitary cell assay refute this argument, and suggest that the porcine PPFF was similar in potency to the equine PPFF, which were both less potent than either the bovine or the ovine preparations. One probable reason for the difference in assay results is that different end-points were used. As discussed in section 1.1.4.1, more factors affect ovulation rate than affect FSH secretion, and the pituitary cell model is thus the preferred assay for inhibin. However, in practical terms, the ovulation rate model is of more interest, as it also takes into account the effects of contaminating activities other than those of inhibin. The anomalous effect of the porcine PPFF is particularly interesting in this respect.

Another reason for the differential assay results could involve species specificity. In the sheep pituitary cell assay, the ovine PPFF was the more potent of those tested. It could be suggested that the bovine preparation would have the highest potency if examined in a bovine pituitary cell assay.

The follicular studies indicate that these preparations did not possess the ability to alter follicular oestradiol secretion, as suggested for some follicular fluid preparations in other animal models (diZerega et al., 1982, 1983a, b, c; Kling et al., 1984; Kigawa et al., 1986a, b). However, since the present study examined extant aromatase activity in a large number of atretic follicles, and not factors such as follicular binding of gonadotrophins or the induction of aromatase activity by FSH, it can be concluded that these preparations may act at the ovarian level.

In conclusion, this study indicates that the partially purified follicular fluid preparations were able to suppress FSH secretion and alter ovulation rate. They are thus suitable materials for the study of inhibin-like activity.

CHAPTER SIX

ACTIVE IMMUNISATION OF CATTLE AGAINST PARTIALLY PURIFIED OVINE FOLLICULAR FLUID

6.1 INTRODUCTION

Active immunisation of sheep against inhibin-enriched fractions of partially purified bovine follicular fluid increased the incidence of multiple ovulations (Henderson et al., 1984c; Cummins et al., 1986a, b) and of twin and triplet foetuses in early pregnancy (O'Shea et al., 1984). There is ample evidence in the literature that injected follicular fluid acts to decrease FSH secretion in cattle as well as in sheep (see section 1.1.4), although at the outset of this work there were no published reports of attempts to immunise cattle against inhibin-rich preparations. Very recently, preliminary results have suggested an increase in ovulation rate in heifers actively immunised against an affinity and FPLC purified fraction of bovine follicular fluid (Sreenan et al., 1987).

The aim of this study was to investigate the effects on ovulation rate of actively immunising heifers against a partially purified fraction of ovine follicular fluid.

6.2.1 Protocol

Twenty-nine Charolais and Simmental cross yearling heifers were divided into four treatment groups of five heifers, and a control group of nine heifers. The treatment animals were immunised against one of two doses of ovine-I PPFF (0.4mg and 4.0mg protein/animal) in NUFA with or without C. parvum; the composition of the emulsion for each group is given in Table 6.1. Additionally, all treatment animals received a single 2.5ml injection of Bord. pertussis subcutaneously in the brisket. The control heifers were not injected. After the priming injection, animals immunised without C. parvum were given two booster injections, and animals immunised with C. parvum were given three booster injections at intervals of four months. The second and third booster injections were devoid of C. parvum and Bord. pertussis. Prior to the third booster injection, the number of control heifers was reduced to six, and one animal from group B was slaughtered because of illness unrelated to the experimental procedures.

Serum samples were taken weekly after the priming and first booster immunisations for antibody titre estimations, and three times weekly after the second and third booster injections to allow antibody titre determinations, and assay of progesterone, and tonic LH and FSH concentrations. Observations for oestrus were made throughout, and the ovulation rate and the number of large follicles (approximately $\geq$10mm in diameter) were determined after each booster

TABLE 6.1

Composition of the emulsion for each animal in each of the treatment groups.

	Immunogen (mg; ml)	NUFA (ml)	Saline (ml)	*C. parvum* (ml)
Group A	0.4; 0.16	1.5	1.5	-
Group B	0.4; 0.16	1.5	0.5	1.0
Group C	4.0; 1.60	1.5	-	-
Group D	4.0; 1.60	1.5	-	1.0*

* Freeze-dried and dissolved in the immunogen before emulsification.

injection, by mid-ventral laparoscopy.

6.2.2 Antibody Characterisation

Antibody production was monitored by double diffusion in agar gels (Ouchterlony and Nilsson, 1978). All gels were stained with Amido Black (Sigma) before being examined. The highest dilution of the antiserum which produced a line of precipitation was defined as the titre; the minimum detectable titre was set at one (neat antiserum). Cross-reactivity of the antisera against follicular fluids from the cow, pig, horse and human was tested in a similar double diffusion assay. The antisera were also tested for cross-reactivity against bovine and ovine whole sera, bovine serum albumin (Sigma), ovine LH (NIH-S18), FSH (NIH-S9), and thyroid stimulating hormone (NIAMMD-9), and bovine prolactin (USDA-B1) and growth hormone (USDA-B1).

To investigate whether the serum from an immunised animal was capable of neutralising the effects of the antigen on ovulation rate in the mouse, the following modifications were made to the bioassay described in Chapter Five. At 0900h on the first day of the assay, groups of mice were injected with 100μl sterile saline or 100μl of a given dilution of serum from an immunised heifer. The serum preparations were incubated with charcoal to remove steroids, and were injected into each mouse immediately before the injection of 150μg antigen.

6.2.3 Hormone Assays

The extraction efficiency of the progesterone assays was approximately 70%, with a sensitivity of 0.12ng/ml. The inter- and intra-assay coefficients of variation over 4 assays were 18.9 and 17.1%, and 22.9 and 14.2%, respectively, for control samples containing 1.5 and 3.0ng/ml. The LH and FSH determinations were each completed in a single assay, with intra-assay coefficients of variation for both assays of approximately 4%, and sensitivity of 0.04ng/tube and 0.5ng/tube, respectively.

6.2.4 Statistics

Hormone data are expressed as geometric means (±s.e.m.), and all other data are expressed as arithmetic means (±s.e.m.). Differences between means were tested by Students t test, except for large follicle populations which were analysed by the Mann-Whitney rank test.

6.3.1 Immunological Response

All heifers immunised with C. parvum (Groups B & D) showed an immune response (Figure 6.1) whereas those without C. parvum (Groups A & C) did not produce any detectable antibodies. The antibody titres attained by the 0.4mg dose group (Group B) following peak titre levels were significantly lower than those of the 4.0mg group (Group D; $P<0.05$). Some animals of both Groups B and D displayed local hypersensitivity reactions at the injection sites, but these regressed without abscess formation.

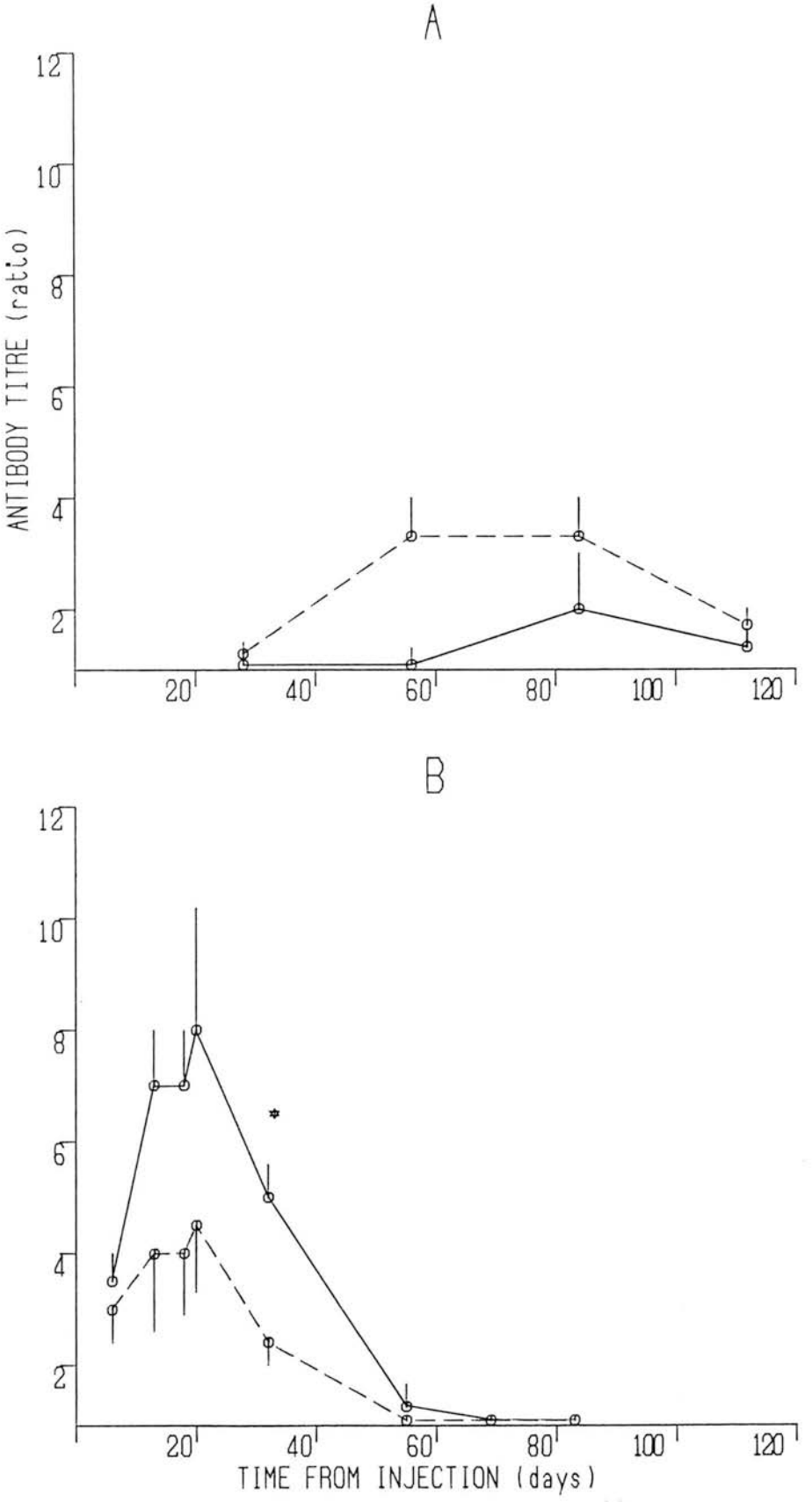
The antisera from Group D animals strongly cross-reacted with ovine follicular fluid and serum, but weakly with bovine and porcine follicular fluids and bovine serum in the double diffusion assay used. No cross-reaction was detected with equine or human follicular fluids, bovine serum albumin, or any of the hormones tested. Immuno-blotting tests of the antisera from Group D heifers indicated that the animals have responded to a large number of the component proteins (R.L.Spooner & R.A.Oliver, unpublished observations). The antiserum tested in the mouse bioassay was taken from heifer 6, at the time of multiple ovulation, but was unable to significantly raise the ovulation rate of the inhibin-treated mice (Table 6.2).

FIGURE 6.1

Mean (± s.e.m.) antibody titres of heifers actively immunised against partially purified ovine follicular fluid after priming (A), and first (B), second (C) and third (D) booster immunisations.

Animals were immunised with 0.4mg (-----) or 4mg (———) protein in Non-Ulcerative Freund's Adjuvant with C. parvum.

Asterisks indicate that the mean titres attained were significantly different between groups ($P<0.05$).


A
B
ANTIBODY TITRE (ratio)
TIME FROM INJECTION (days)
2
4
6
8
10
12
20
40
60
80
100
120
*

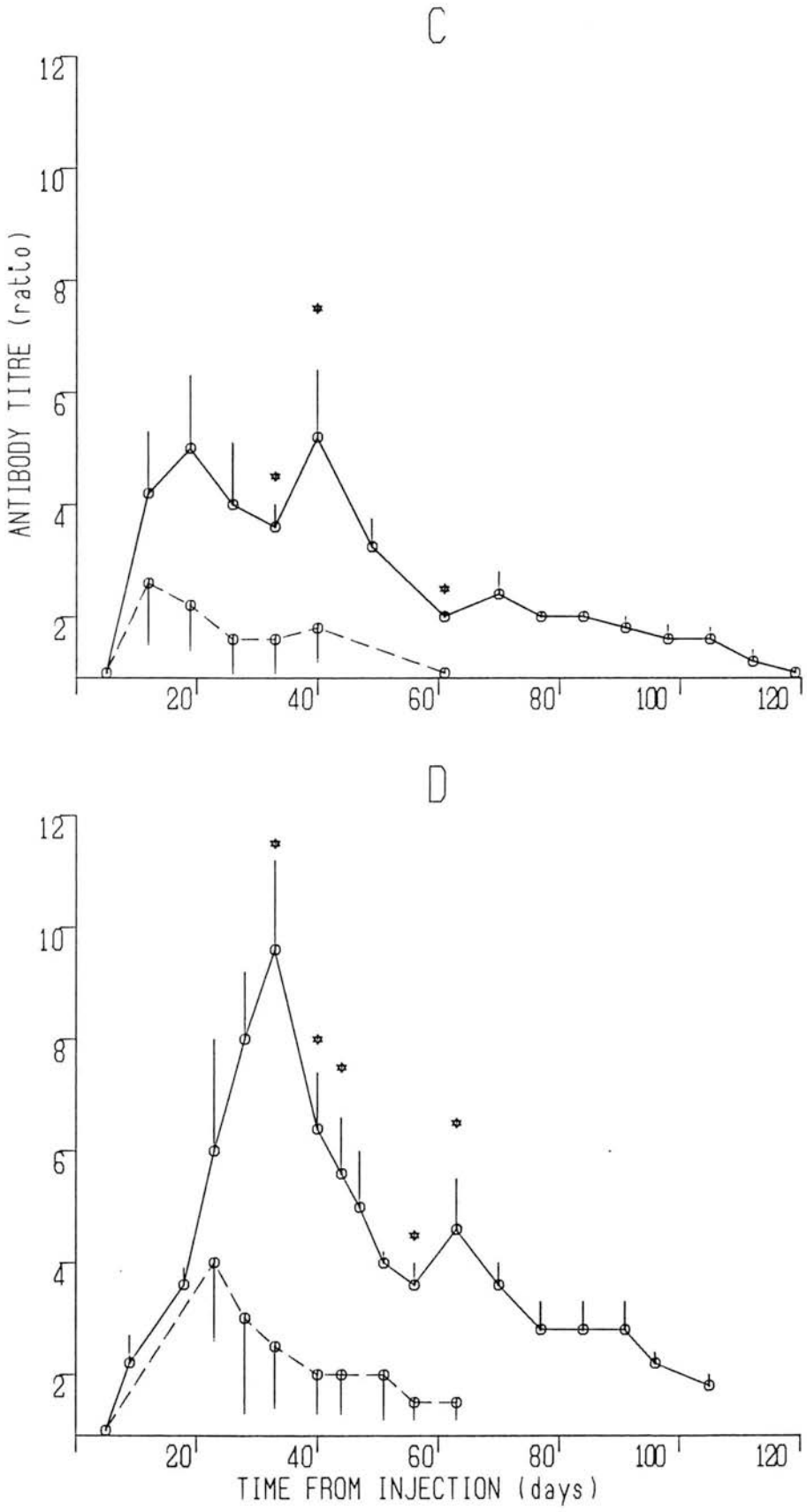

C
D
ANTIBODY TITRE (ratio)
12
10
8
6
4
2
20
40
60
80
100
120
TIME FROM INJECTION (days)

TABLE 6.2

Ovulation rate of mice injected with saline, ovine-I PPFF / PPFF dilutions and of serum from an immunised heifers showing multiple ovulations.

The data are expressed as arithmetic means (±s.e.m.).

Antigen (μg)	Antiserum dilution	Number of mice per dose	Ovulation rate
Nil	Nil	10	9.1 ± 1.3^{a}
150	Nil	10	2.7 ± 0.9^{b}
150	1:1	10	3.2 ± 1.2^{b}
150	1:10	10	3.7 ± 1.1^{b}
150	1:100	10	4.4 ± 1.3^{b}

a, b
Means with different superscripts down a column differ ($P<0.05$)

6.3.2 Ovarian Response

Immunisation without C. parvum (Groups A & C) had no effect upon ovulation rate or the number of large follicles at any period of the experiment. Immunisation with C. parvum (Groups B & D) had no effect on ovarian activity after the priming or first booster immunisations. However, following the second booster injection, there was a dose-dependent increase in ovulation rate; one heifer in Group B (0.4mg protein) showed a double ovulation, whereas in Group D (4.0mg protein), two heifers showed double ovulations and one heifer displayed a triple ovulation (Table 6.3). This response was observed for one oestrous cycle only. The difference in ovulation rate between Group D and the control heifers after the second booster injection was not significant by Student's t test owing to the small numbers involved, but the probability of observing three out of five animals with double ovulations (and assuming a twinning rate as high as 8%) is less than 0.005.

After the third booster injection, one double ovulation was seen in a heifer from Group D, however, two heifers from Group D and one from Group B exhibited oestrus without ovulating (Table 6.3). Forty-nine observations were made on the control heifers and only one double ovulation was observed.

There were no differences between the number of large follicles observed in control and immunised heifers after the first or second booster injections. After the third booster injection, the heifers in Group D possessed a higher number of large follicles than the control heifers (1.2 ± 0.2 vs 0.6 ± 0.2 large follicles per heifer, Group D and control heifers, respectively; $P<0.05$), but not so for Group B heifers (0.7 ± 0.2 large follicles per heifer). The Group D

TABLE 6.3

Number of corpora lutea observed in successive oestrous cycles for groups B and D following second and third booster injections.

Group	Animal identity	After second booster	After third booster
B	1	1, 1	0, 1, 1
	2	1, 1	*
	3	1, 2	1, 1
	4	1, 1	1, 1
	5	1, 1	1, 1, 1
D	6	3, 1, 1	1, 0, 2, 0, 1
	7	2, 1, 1	0, 1, 1
	8	2, 1, 1	1, 1, 1, 1
	9	1, 1, 1	1, 1, 1
	10	1, 1, 1	1, 1, 1, 1

A score of 0 indicates anovulation.

* Heifer 2 was slaughtered shortly before the third booster was due to be given.

heifers also demonstrated a significantly shorter mean oestrous cycle length than that of the control heifers (19.1±0.3 vs 20.0±0.3 days for Group D and control heifers, respectively; $P<0.05$).

There was no detectable correlation between ovulation rate and antibody titre at oestrus in any heifer from Group D (Figure 6.2).

6.3.3 Endocrine Response

There were no statistical differences in LH concentrations between Group B or D heifers and the control heifers after second or third booster injections. There was a trend for LH concentrations to rise immediately after the second booster injection, however, at this time four of the five animals in both treatment groups were in the follicular phase of the oestrous cycle, whereas there was only one follicular phase control heifer; thus the gonadotrophin trends were probably a result of the stage of the cycle.

Five weeks after the second booster injection, the mean FSH concentrations in Group B heifers were significantly higher than those of the control heifers (Table 6.4). This effect was transient, and there was no such difference between Group D and control heifers. The only abnormal progesterone profiles were obtained from the heifers which were anovulatory after the third booster injection.

FIGURE 6.2

Relationship between antibody titre and ovulation rate at first oestrus after the second booster injection in each of the five heifers immunised against 4mg protein with C. parvum (Group D).

The heifers shown in red displayed multiple ovulations.

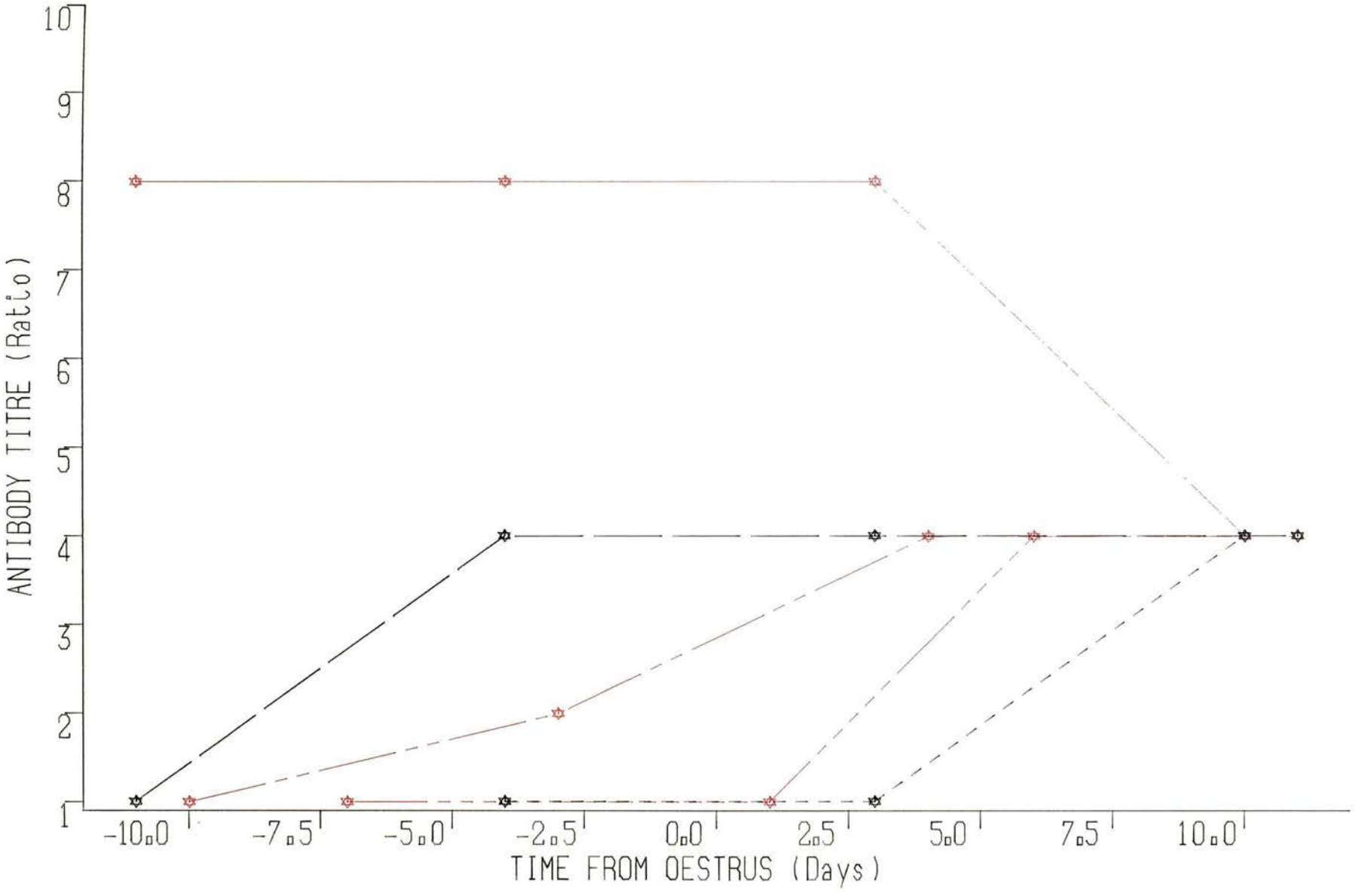

ANTIBODY TITRE (Ratio)
10
9
8
7
6
5
4
3
2
1
-10.0
-7.5
-5.0
-2.5
0.0
2.5
5.0
7.5
10.0
TIME FROM OESTRUS (Days)

TABLE 6.4

Mean FSH concentrations (±s.e.m.) in Group B, D and control heifers after the second booster injection.

Time (weeks)	FSH (ng/ml)		
	Group B	Group D	Control
0	26.9± 8.5	14.4± 4.6	18.4± 5.8
1	38.0±12.0	44.9±16.8	33.2±14.4
2	35.2±10.4	40.0±10.8	19.1± 8.3
3	22.1± 7.0	32.6±10.3	15.4± 4.9
4	37.8±12.0	13.8± 4.4	20.4± 6.5
5	39.8±12.6 *	18.7± 5.9	9.0± 2.9
6	20.9± 6.6	18.0± 5.7	20.1± 6.4

* Group B significantly higher than control group ($P<0.05$)

6.4 DISCUSSION

This study indicates that active immunisation of heifers against partially purified ovine follicular fluid can increase ovulation rate in cattle. The absence of superovulation or prolonged anoestrus in this present study suggests that this may be a more suitable method for the induction of twinning than active immunisation against gonadal steroids (see Chapter 3).

These effects were, however, of a transient nature. There were no effects of the immunisation on ovarian function prior to the second booster injection, and the increase in ovulation rate was observed in one oestrous cycle only. A subsequent booster immunisation did not significantly influence ovulation rate, but did increase the number of large follicles seen on the ovary. It is not clear why the multiple ovulations only occurred for one cycle, or why a further booster immunisation did not elicit the same response. In the published sheep studies, the booster injections were given at monthly intervals (Cummins et al., 1986a); however, in the present study, antibody titres appeared no lower after the third booster injection than after the second booster injection.

An increase in the number of large follicles has also been reported for inhibin immunised sheep (Cummins et al, 1986a). The reason that an increased number of large follicles did not result in an increased number of ovulations after the third booster injection is not clear. The mean length of the oestrous cycle was shorter in immunised than in control heifers at this time, but is unlikely to have affected the number of follicles ovulating because, although significant, it still lies within the expected range for the cow (Lasley and Bogart, 1943).

The mechanisms by which the numbers of corpora lutea and large follicles can be increased by inhibin immunisation are not known, largely because the actions of inhibin itself have yet to be clarified. If inhibin affects FSH secretion directly, it must reach the hypothalamus/pituitary gland via the peripheral circulation; immunisation would be expected to neutralise this circulating activity and hence increase FSH secretion and ovulation rate. The lack of a significant effect of the treatment on gonadotrophin secretion in Group D heifers does not support this hypothesis. Inhibin activity in the peripheral blood of the large domestic species has only recently been described (Tsonis et al., 1986a), although the lymphatic system may also be important for inhibin transportation (Findlay et al., 1986). The sheep studies previously reported (Henderson et al., 1984c; O'Shea et al., 1984) have also indicated that active immunisation against inhibin does not affect gonadotrophin secretion. Cummins et al. (1986a) pointed out that differences in FSH secretion after immunisation of ewes could be observed in a homologous assay system, but not in a heterologous assay. This may reflect differences in the molecular species of FSH secreted, as reported in the rat (Foulds & Robertson, 1983; Chappel & Ramaley, 1985), hamster (Chappel, 1981) and monkey (Chappel et al., 1984b).

The antibodies in the present study may have increased the number of large follicles on the ovaries by rescuing some from atresia. Follicular fluid preparations have been reported to cause granulosa cell degeneration and to decrease aromatase enzyme activity in the immature rat and mature pig ovary (DiZerega et al., 1983a, b; Chari et al., 1985), both indicators of follicular atresia. However, a direct effect of these immunisations on

gonadotrophin secretion cannot be excluded until more detailed examinations of pulsatile gonadotrophin secretion have been conducted.

The physiology of inhibin can be clarified once pure preparations of the hormone are widely available. Recent reports have revealed that the β-subunits of the two inhibin molecules present in follicular fluid (see section 1.1.4.2) can dimerise to form a FSH releasing protein of molecular mass 24-28kDa (Ling et al., 1986a, b; Vale et al., 1986). It is possible that antibodies have been raised to both FSH suppressing and releasing proteins in the present study, and that the resultant antagonistic effects may obscure changes in FSH secretion.

There was a marked difference in the efficacy of adjuvants employed in the present study, which contrasts with the previous immunisation experiment (Chapter 3). Animals immunised without C. parvum did not produce any detectable antibodies, whereas those immunised with C. parvum exhibited high antibody titres without the formation of necrotic lesions usually associated with the use of Mycobacterium tuberculosis. It is suggested that this adjuvant should be employed in cattle when high titres are desired from dilute or complex protein antigens.

In summary, these results have shown that a controlled increase in ovarian activity and ovulation rate can be achieved in cattle by actively immunising against an inhibin-rich fraction of ovine follicular fluid. This increase was not associated with anoestrus or the formation of ovarian cysts, and offers considerable potential for increasing the fecundity of beef cattle.

CHAPTER SEVEN

ACTIVE IMMUNISATION OF CATTLE AGAINST PARTIALLY PURIFIED OVINE, PORCINE AND EQUINE FOLLICULAR FLUIDS

7.1 INTRODUCTION

The previous experiment indicated that active immunisation against partially purified ovine follicular fluid could increase the incidence of twin and triplet ovulations in cattle. The mechanism of this action is unclear, but is believed to be a result of neutralising inhibin-like activity in the circulation or within the ovary itself. The published results of sheep immunisation suggest that there are no clear effects on FSH secretion (Henderson et al., 1984c; O'Shea et al., 1984). It is unknown if the ovine preparation is an optimum source of inhibin-like activity for these studies.

The aim of this experiment was to address these questions by examining in detail gonadotrophin and steroid secretion in cattle after active immunisation against partially purified ovine, porcine or equine follicular fluids.

7.2.1 Protocol

Twenty-one Hereford-Friesian yearling heifers were divided into four groups, and were actively immunised against either ovine-II (Group O; n=5), equine (Group E; n=6) or porcine (Group P; n=6) PPFF, or injected with adjuvant alone (Group C; n=4). Each immunised heifer received 4mg protein in 5ml Non-Ulcerative Freunds Adjuvant, with 1ml *C. parvum* and the antigen incorporated into the aqueous component of the adjuvant (the composition of each emulsion is given in Table 7.1). In addition, each treated animal received 2.5ml *Bord. pertussis* injected subcutaneously into the brisket. The antigens were characterised in Chapter Five.

Four months after the priming immunisation, all heifers were given the first booster injection. This, and the second booster injection, were devoid of *C. parvum* and *Bord. pertussis*.

Six months after the first booster injection, the oestrous cycles of all animals were synchronised by two injections of PGF2α eleven days apart. The second booster was injected on Day 15 of the synchronised cycle.

Observations for oestrus were made following each immunisation, and the ovulation rate and the number of large follicles ($\geq$10mm diameter) were monitored by sublumbar laparoscopy.

TABLE 7.1

Composition of the emulsion for each animal in each of the treatment groups.

	Immunogen (mg; ml)	NUFA (ml)	Saline (ml)	*C. parvum* (ml)
Group P	4.0; 0.9	2.9	0.9	1.0
Group E	4.0; 1.7	2.9	0.1	1.0
Group O	4.0; 1.4	2.9	0.4	1.0
Group C	Nil	2.9	1.8	1.0

7.2.2 Blood Sampling

Blood samples were taken fortnightly after the first booster injection, and weekly after the second booster injection, for antibody titre determinations by double diffusion in agar gels (Ouchterlony & Nilsson, 1978).

Samples were taken three-times weekly from the date of the second PGF2α injection, prior to the second booster injection, for progesterone analysis. To determine the pattern of LH secretion, blood samples were taken every 10 min for 12 hr on Day 13-14 of the cycle following second booster injection, and again for 6 hr on Day 19-20 of the cycle. During these intensive sampling periods, samples were taken hourly for mean FSH determinations, and three samples were taken within each period for mean oestradiol determinations. This protocol is illustrated in Figure 7.1.

7.2.3 Hormone Assays

The LH estimations were completed in 10 assays with a mean sensitivity of 0.04ng/tube. The intra-assay coefficient of variation (calculated from 20 duplicate pairs) was 4%, and the inter-assay coefficient of variation was 11%. The FSH determinations were completed in two assays, with a sensitivity of 0.6ng/tube, and intra- and inter-assay coefficients of variation of 13%.

The progesterone analyses were completed in three assays, with a mean extraction efficiency of 60%. The sensitivity of the assays was 0.11ng/ml and intra-assay coefficient of variation (calculated

from 20 duplicate pairs) of 3.5%. The inter-assay coefficients of variation for plasma samples containing 1.5 and 4ng/ml were 12% and 4%, respectively. The oestradiol determinations were completed in one assay with an extraction efficiency of 64%, and intra-assay coefficient of variation (calculated from 20 duplicate pairs) of 7.7%. The sensitivity of this assay was 0.6pg/tube.

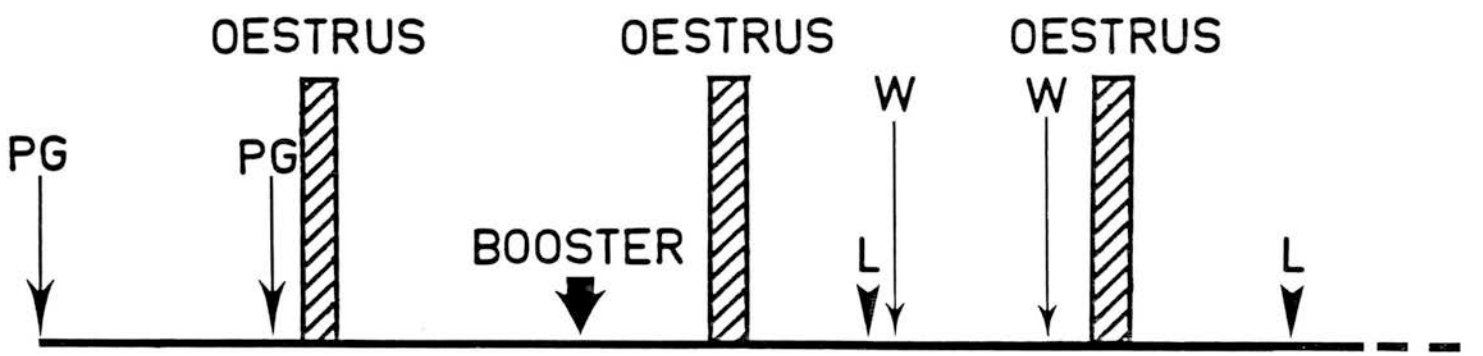
7.2.4 Statistics

All hormone data are expressed as geometric means (±s.e.m.). LH pulse analyses were performed by the pulse programme described in Chapter Two. The effects of the treatments upon hormone secretion were examined by analysis of variation, with corrections for multiple comparisons.

FIGURE 7.1

Design of the immunisation experiment.

Oestrous cycles were synchronised with two consecutive injections of prostaglandin-F2α (PG), and all animals received the second booster injection on Day 15 of the synchronised cycle (BOOSTER). In the following cycle, all animals were blood sampled intensively (every 10 min) for 12 and 6 hr in the luteal and follicular phases of the cycle, respectively (W). All animals also underwent laparoscopy in the luteal phase (L) for three successive cycles.


OESTRUS
OESTRUS
OESTRUS
PG
PG
BOOSTER
W
W
L
L

7.3.1 Immunological Response

The antibody titres observed in the present experiment were generally higher than those achieved in Chapter Six. The peak titres produced after the second booster injection in Group P were significantly higher than those of Groups E and O (Figure 7.2). The control heifers did not show antibody titres above minimum detectable.

Some heifers from each group showed local hypersensitivity reactions, but these regressed without abscess formation. Immunoblotting tests revealed that all animals were responding to a large number of proteins within the PPFF preparations (R.L.Spooner & R.A.Oliver, unpublished observations).

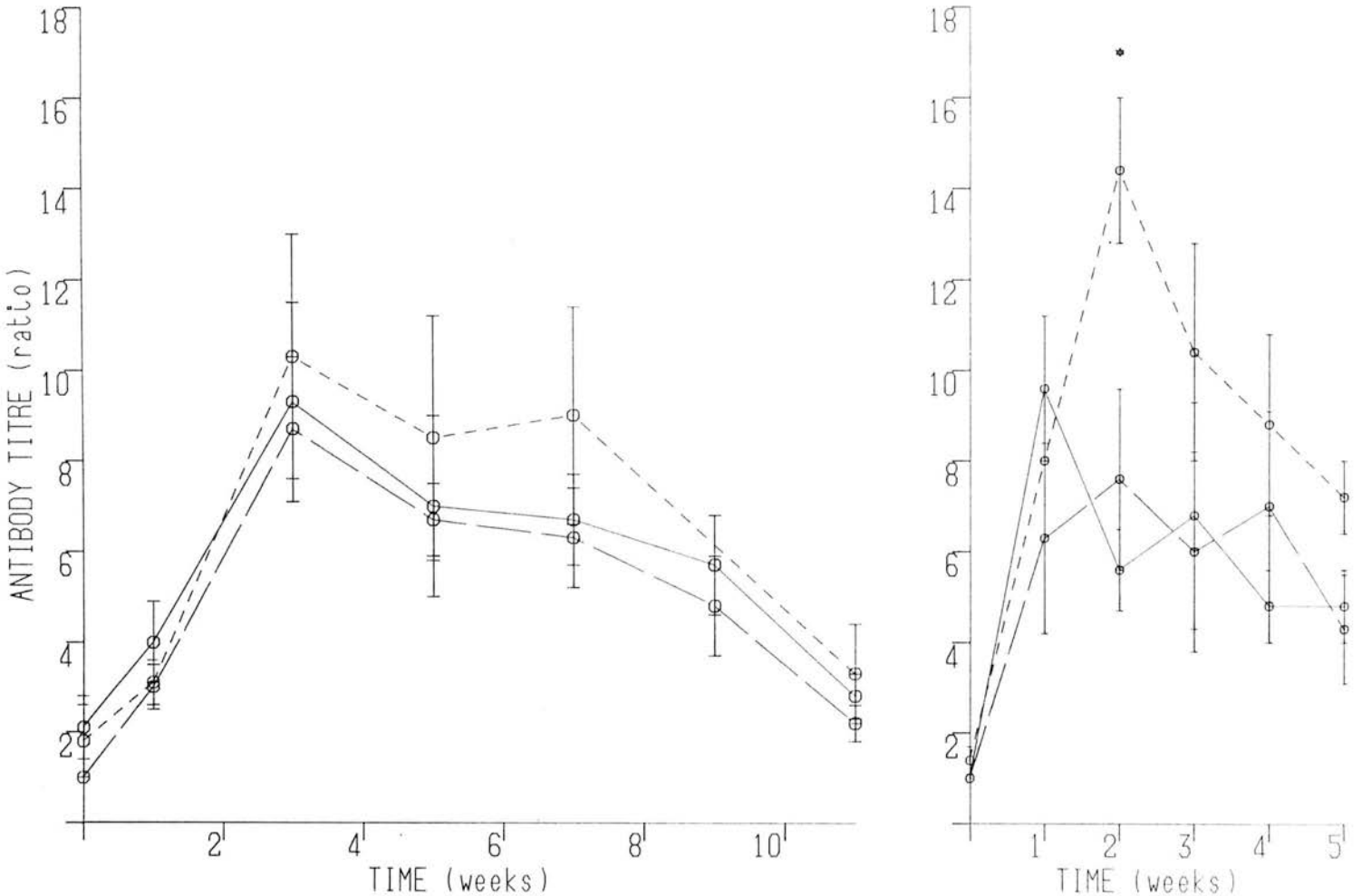
7.3.2 Ovarian Response

There was no effect of any treatment upon ovulation rate after the primary or first booster immunisations. After the second booster injection, one of the heifers from Group O showed a double ovulation. There was no effect upon either the number of large follicles in the ovaries or oestrous cycle length.

FIGURE 7.2

Mean (± s.e.m.) antibody titres in heifers actively immunised against partially purified ovine (———), equine (— — —) or porcine (----) follicular fluids, after first and second booster injections.

The titres are expressed as the highest dilution of antiserum that produced a line of precipitation after double diffusion in agar gels. The asterisk indicates that the mean peak titre in the porcine follicular fluid immunised heifers was significantly higher than that of the other immunized heifers ($P<0.05$)


ANTIBODY TITRE (ratio)
18
16
14
12
10
8
6
4
2
2
4
6
8
10
TIME (weeks)
18
16
14
12
10
8
6
4
2
1
2
3
4
5
TIME (weeks)

7.3.3 Endocrine Responses

The mean LH and FSH concentrations observed in each group are depicted in Figures 7.3 and 7.4. The mean LH concentrations of all immunised heifers were significantly higher than those of the control heifers, during both phases of the oestrous cycle. A significant increase in mean FSH concentrations was apparent only during the follicular phase of the porcine PPFF immunised heifers.

There were no differences in pulsatile LH secretion, in either phase of the cycle, between Groups E or O and Group C heifers (Figure 7.5). During the follicular phase, LH pulse frequency was significantly lower, and pulse amplitude significantly higher in Group P than in Group C ($P<0.05$; Figure 7.5).

During the luteal phase of the cycle, there were no differences between groups in oestradiol (Table 7.2) or progesterone secretion (3.5 ± 0.2, 3.4 ± 0.3, 3.4 ± 0.3 and 2.9 ± 0.2 ng/ml for Groups P, E, O and C, respectively; $P<0.05$). During the follicular phase of the cycle, however, mean oestradiol concentrations in Groups P, E and O were significantly lower than those of Group C ($P<0.05$).

FIGURE 7.3

Geometric mean (± s.e.m.) plasma LH concentrations in luteal and follicular phases of the oestrous cycle of heifers immunised against partially purified porcine (P), equine (E) or ovine (O) follicular fluids, or injected with adjuvant alone (C).

Asterisks indicate groups significantly different from controls ($P<0.05$).

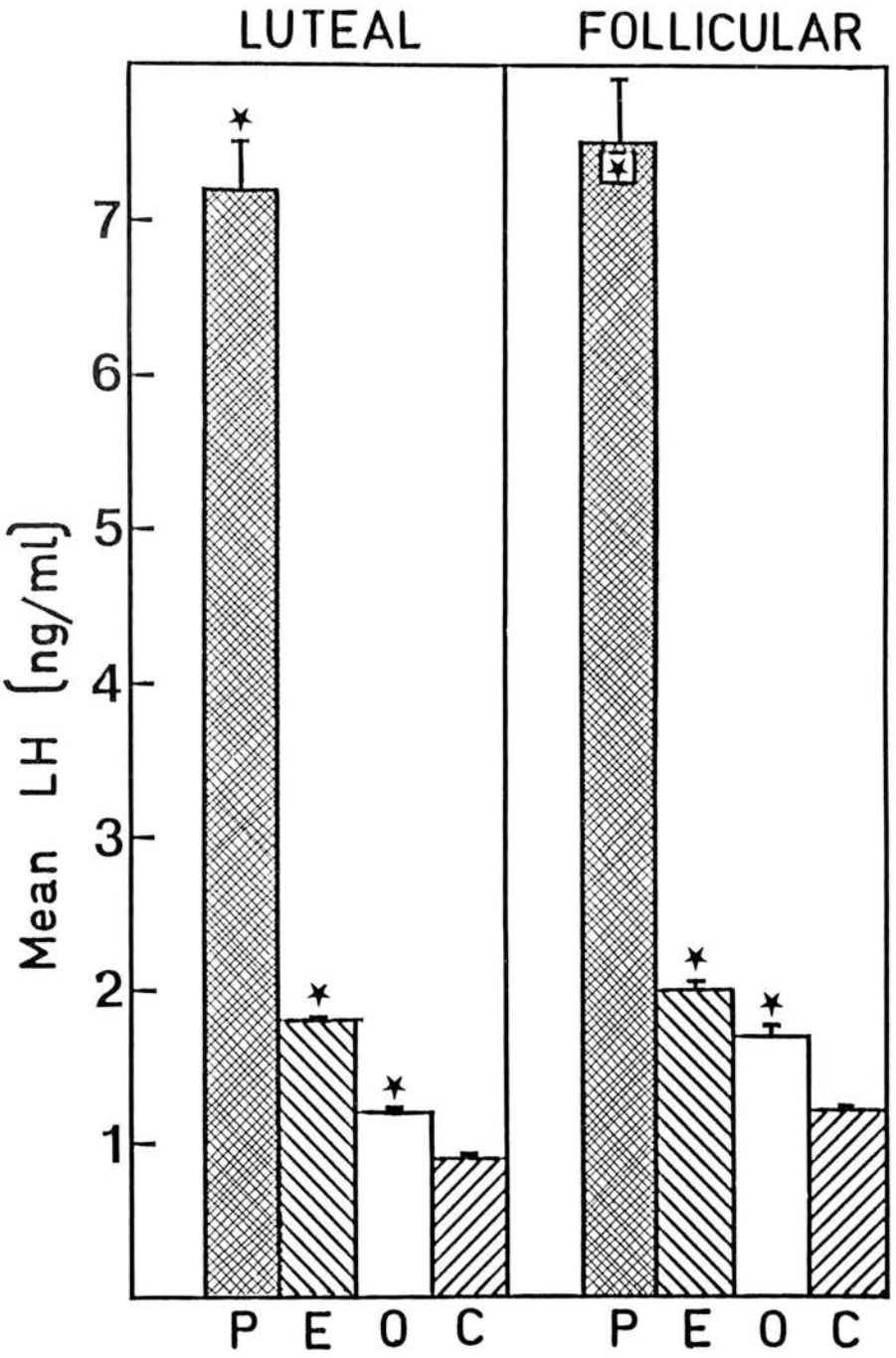

LUTEAL
FOLLICULAR
Mean LH (ng/ml)
7
6
5
4
3
2
1
P E O C
P E O C

FIGURE 7.4

Geometric mean (± s.e.m.) plasma FSH concentrations in luteal and follicular phases of the oestrous cycle of heifers immunised against partially purified porcine (P), equine (E) or ovine (O) follicular fluids, or injected with adjuvant alone (C).

The asterisks indicates that Group P is significantly different from Group C ($P<0.05$).

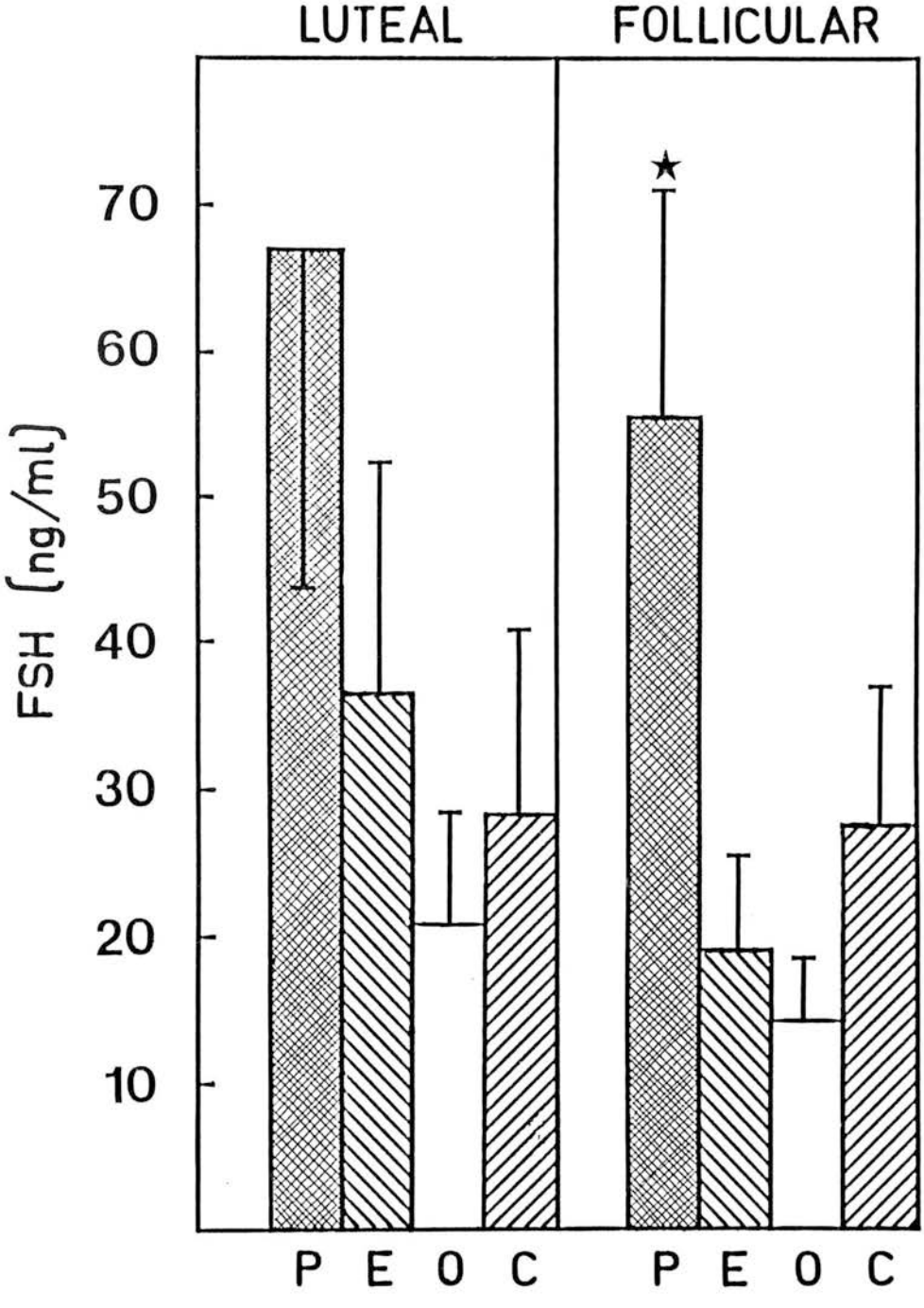
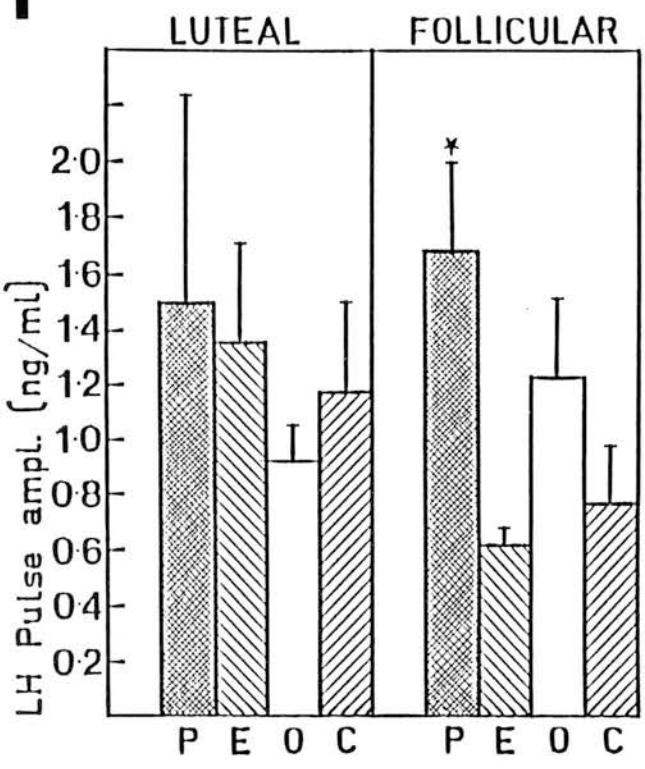

LUTEAL
FOLLICULAR
FSH (ng/ml)
70
60
50
40
30
20
10
P E O C
P E O C

FIGURE 7.5

Geometric mean (± s.e.m.) plasma LH pulse amplitude (i) and pulse frequency (ii) in luteal and follicular phases of the oestrous cycle of heifers immunised against partially purified porcine (P), equine (E) or ovine (O) follicular fluids, or injected with adjuvant alone (C).

Asterisks indicate groups significantly different from controls ($P<0.05$).

i


LUTEAL
FOLLICULAR
LH Pulse ampl. (ng/ml)
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
P E O C
P E O C

ii

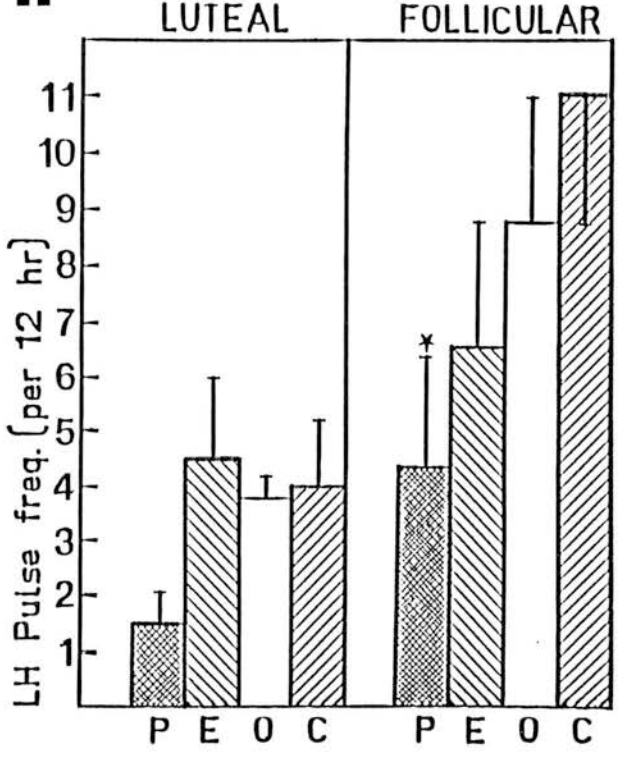

LUTEAL
FOLLICULAR
LH Pulse freq. (per 12 hr)
11
10
9
8
7
6
5
4
3
2
1
P E O C
P E O C

TABLE 7.2

Geometric mean (±s.e.m.) oestradiol concentrations (pg/ml) during the luteal and follicular phases of the oestrous cycle of heifers immunised against porcine (Group P), equine (Group E) or ovine (Group O) PPFF. Control heifers (Group C) were given adjuvant alone.

Group [+]	Luteal phase	Follicular phase
Group P (6)	1.0±0.2[a]	2.9±0.5[b]
Group E (6)	1.2±0.2[a]	2.8±0.2[b]
Group O (5)	1.0±0.1[a]	2.6±0.4[b]
Group C (4)	0.8±0.1[a]	6.5±1.3[c]

a,b,c Means with different superscripts down a column differ ($P<0.05$)

+ Numbers of heifers in each group are shown in brackets

7.4 DISCUSSION

The most striking result of this experiment is the 7-fold increase in circulating LH concentrations in the porcine PPFF immunised heifers. This increase was not accompanied by an increase in LH pulse frequency or in ovulation rate.

An increase in mean LH concentrations, pulse frequency and pulse amplitude has been reported in sheep following the infusion of steroid-free bovine follicular fluid (Wallace & McNeilly, 1986). This was attributed to reduced negative feedback effects of oestradiol occurring as a result of the follicular fluid-induced reduction in circulating FSH concentrations. Whilst a significant reduction in circulating oestradiol concentrations during the follicular phase of the cycle was observed in the present study, this does not satisfactorily explain the elevated LH secretion in the porcine PPFF immunised heifers. The decreased oestradiol secretion was not different between Groups P, E and O, despite significant differences in LH and FSH secretion between these groups. Also, a decrease in oestradiol feedback would increase LH pulse amplitude and frequency, as well as mean concentrations, as observed in sheep (Wallace & McNeilly, 1986) and cattle (Chapter Four); this was not observed in the present study.

Collectively these data suggest that porcine follicular fluid contains a non-steroidal factor which can inhibit basal LH secretion in the cow. This supports the concept of several pituitary pools of LH, and provides evidence for a control system specifically aimed at basal LH secretion. An inhibitor of this nature has not previously been proposed. Most reports in the literature demonstrate the selective suppression of FSH by whole porcine follicular fluid

(monkeys: Channing et al., 1979, 1981a; rats: Charlesworth et al., 1984; sheep pituitary cells; Huang & Miller, 1984) and monkeys immunised against whole porcine follicular fluid did not show elevated LH concentrations (Channing et al., 1982b). However, porcine follicular fluid has been shown to decrease basal FSH and LH secretion in ovariectomised monkeys (Schenken et al., 1984), and to reduce mean concentrations and pulse amplitude of LH in oestrogen-implanted, ovariectomised rats (Babu et al., 1986). The physiological importance of this factor is obscure, but its presence in the follicular fluid of a polytocous species is of interest.

The effects of the present treatment on FSH secretion were limited to the porcine PPFF immunised heifers during the follicular phase of the cycle. This was not related to ovarian activity, as the only recorded double ovulation occurred in a heifer immunised against ovine PPFF. This ovulatory response is lower than that observed in Chapter Six, and may have been a chance occurrence. The reason for this difference is unclear, as the two ovine preparations were equipotent in the sheep pituitary cell bioassay (see Chapter Five), but it indicates that inconsistent ovarian responses in cattle are not confined to steroid immunisations.

The lack of a significant increase in circulating FSH concentrations in the ovine PPFF immunised heifers is in agreement with the results in sheep (Henderson et al., 1984c; O'Shea et al., 1984). Further, during the follicular phase of the cycle, heifers of Groups E and O secreted significantly lower steroid concentrations than the control heifers, but showed mean FSH concentrations that were not higher than those of the control heifers. Thus native inhibin may have been suppressing FSH concentrations in the present study.

In summary, these results indicate that the current immunisation did not neutralise circulating inhibin-like activity, but did significantly suppress ovarian oestradiol secretion. This may have been a direct effect upon the ovary. Also, strong evidence has been produced for a non-steroidal inhibitor of basal LH secretion in porcine follicular fluid. That this putative hormone has a physiological role in the control of LH secretion is uncertain, but it may be an important local regulator of ovarian function.

CHAPTER EIGHT

GENERAL DISCUSSION

The immunisation studies reported in this thesis collectively suggest that, in the cow, ovulation rate does not respond consistently to treatments so far designed to alter gonadotrophin secretion. Immunisation against testosterone (Chapter Three) elevated LH secretion and caused prolonged periods of anoestrus. This effect was probably exacerbated by the presence of ovarian cysts. However, several animals did show oestrus coupled to a high incidence of multiple ovulation. The increased ovulation rate was not observed to be correlated with antibody titre or the pattern of hormone secretion.

Active immunisation against partially purified ovine follicular fluid increased ovulation rate without altering gonadotrophin secretion in one study (Chapter Six), although a subsequent study (Chapter Seven), with a similarly prepared antigen of equal inhibin-like activity, showed that LH secretion was increased but ovulation rate was unaffected.

These data suggest that the ovary of the cow possesses an important intra-ovarian control mechanism. The proteins present in follicular fluid may be involved in this mechanism. One indirect line of evidence for this is the seven-fold increase in basal LH secretion obtained following immunisation against porcine follicular fluid (Chapter Seven). This increase could not be explained by changes in steroid feedback from the ovary, and a direct effect of porcine follicular fluid on LH secretion is not supported in the literature. The action upon LH secretion may thus be pharmacological, or the result of an ultra-long feedback loop involving the ovary as an intermediate step.

Since immunisation against inhibin-enriched ovine follicular fluid increased ovulation rate without increasing tonic

gonadotrophin secretion (Chapter Six), this activity, too, may have had a direct effect in the ovary. To support this, in the cow at least, the results of Chapter Four suggest that physiological concentrations of the gonadal steroids are capable of maintaining LH and FSH secretion to within the range observed during the luteal and follicular phases of the oestrous cycle, without the need for inhibin.

Further evidence for the predominant control of FSH secretion by gonadal steroids is available for the cow. In sheep, circulating FSH concentrations are significantly lower during the follicular phase of the oestrous cycle than in the luteal phase (Baird, 1983), and this effect has been attributed to increased follicular secretion of inhibin during the follicular phase. In cattle, however, no consistent decline in FSH concentrations has been reported in the literature (Schams & Schallenberger, 1976; Schallenberger et al., 1984; Walters & Schallenberger, 1984; Walters et al., 1984; Peters, 1985) nor observed in the control heifers used in this thesis (Chapters Three and Seven).

It may be that comparison of the luteal with the follicular phase of the oestrous cycle is inappropriate. A recent report suggests that the ovulatory follicle in the cow is selected at luteolysis (Ireland & Roche, 1987). The endocrine patterns observed in the present studies have not considered this dynamic phase of the oestrous cycle, although earlier work suggests that intra-ovarian mechanisms may be of importance; anaesthetised cattle did not display a rise in tonic LH secretion at luteolysis, but oestradiol concentrations still rose as progesterone concentrations fell (Fogwell et al., 1978).

It is also possible that the changes in FSH secretion required

to raise ovulation rate are sufficiently subtle to escape detection in present radioimmunoassay systems. Brown (1978) proposed that changes in mean FSH secretion of only 10% are required for normal ovarian function in humans.

Only one of the treatments in this thesis increased mean FSH secretion in the intact heifer (Chapter Seven) and this was not associated with an increase in ovulation rate, although this treatment also increased mean LH concentrations. The correlation between ovulation rate and FSH secretion is not firmly established, either in the cow or in the sheep (section 1.2.3).

It is tempting to speculate that, given 'adequate' circulating LH and FSH concentrations, the intra-ovarian mechanism ultimately determines ovulation rate in the cow. The components of this mechanism would probably not fulfil the classical definition of a hormone, and would not directly influence gonadotrophin secretion. Rather, these components would ensure that the ovary responded to the peripheral endocrine milieu by producing only one ovulatory follicle. These components, proteins, peptides or steroids, could thus be regarded as guardians of the single ovulatory follicle, and, for ease of reference, the name 'tutelin' is suggested for these putative compounds (from the Latin tutela, meaning 'guard').

Future work should be directed at isolating, purifying and characterising these tutelins, as they may hold the key to increased prolificacy in the large domestic and wild species.

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